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THE PROTECTIVE VALUE OF THE Vi AND THE O ANTIBODY IN RELATION TO THE VIRULENCE OF STRAINS OF *BACT. TYPHOSUM*.

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IN the course of experiments on the extraction of antigenic substances from *Bact. typhosum* (Henderson and Morgan, 1938), the protective value of the Vi and the O antibody produced in the rabbit by immunization with the various extracted substances was estimated by a passive protection test in mice. Mucin was used in these experiments for enhancing the lethal action of the typhoid bacillus. This method brought to light several interesting problems concerning the relative virulence of different strains of *Bact. typhosum*, and the influence that variation in virulence exerts on the protective value of the Vi and the O antibody. The present paper deals with these problems, and describes experiments which help to define more clearly the function of the Vi and the O antibody in the control of experimental typhoid infection in the mouse.

METHODS.

For details of the methods reference should be made to the paper by Henderson and Morgan (1938).

Strains of Bact. typhosum *employed.*—These were representative of the O-agglutinable or O strains, the O-inagglutinable or O + Vi strains, and the so-called rough Vi strains ; they were maintained in sub-culture by methods previously described.

Antibacterial sera used in the protection experiments.—Anti-O and anti-Vi sera of adequate agglutinin titre were produced in the rabbit by means of the technique already described. Rabbits immunized with living suspensions received an initial dose of 500 million organisms given intravenously as a saline suspension prepared from an 18–24-hour agar-slope culture ; subsequent doses were gradually increased to 1000 million organisms and this amount was rarely exceeded.

The estimation in vitro *of the O and the Vi antibody in the experimental sera.*—The agglutination and the agglutinin-absorption tests were carried out as described in the paper indicated above.

The passive protection test in the mouse.—Two methods of test were used which differed only in the form in which the test dose was prepared, that is, whether mucin was added to the dose or not. For both methods mice of mixed breed weighing 18–22 g. were selected, and the dose of serum, which was adjusted to a volume of 0·5 c.c. with saline, was given intravenously 18–24 hours prior to the test dose; the test suspension of organisms was prepared from an 18–24-hour agar-slope culture suspended and diluted in a trypsin-digest broth to the required density. When mucin was used 0·05 c.c. of the required broth dilution of organisms was suspended in 0·45 c.c. of a 5 per cent. solution of it and injected intraperitoneally. Alternatively, the required number of organisms was suspended finally in a total volume of 0·5 c.c. of trypsin-broth and immediately injected intraperitoneally.

Preparation of the mucin suspension.—Granular mucin "Type 1701–W" obtained from the Wilson Laboratories, Chicago, was used. This material was utilized by Miller (1935) for enhancing the virulence of the meningococcus for mice, and was recommended by Rake (1935) for use in similar experiments with *Bact. typhosum*. 5 g. of mucin are suspended in 100 c.c. of freshly prepared distilled water in the following manner: The mucin is ground to a fine powder in a mortar; 20 c.c. of the water is added and thoroughly mixed. After standing for 30 minutes another addition of 20 c.c. of water is made, and the whole is mechanically mixed in the mortar at intervals for one hour. Successive additions of 20 c.c. of water are made at intervals of 20 minutes. The suspension is autoclaved at 15 lb. pressure for 15 minutes and is then gently shaken and allowed to stand for 5–10 minutes, when a granular deposit collects at the bottom of the flask. The supernatant suspension is removed, and the pH adjusted to 7·3 with N/10 NaOH. Sterility tests are made and the material is stored at $\pm$ 1° C. ready for use. In this state the preparation gives satisfactory results for at least 10 days.

RESULTS.

In the earlier experiments referred to above the test strain of *Bact. typhosum* used for the experimental infection of mice was the fully virulent strain Ty. 2, which possesses both the Vi and the O antigen. When mucin was added to the test suspension the average lethal dose (a.l.d.), representing a 50 per cent. mortality rate contained approximately 500 bacilli. By this method the protective action of a Vi antibody and of an O antibody was clearly established. The high efficiency exhibited by both types of serum formed a striking contrast to the relatively low activity of the Vi antibody and the complete absence of protective action of the O antibody as recorded by Felix and Pitt (1934). These workers used the same test strain (Ty. 2), but without mucin as an adjuvant in the test dose; this, therefore, contained 1000 to 10,000 times the number of organisms in an average lethal dose. It was of interest to compare the two methods of test simultaneously in order to estimate with some accuracy the degree of discrepancy in the protective values of the experimental sera. Table I summarizes the results of one passive-protection test. In this experiment an estimate was made of the protective value of a Vi serum which had been prepared by immunizing rabbits

with a living rough Vi suspension (T. 6 S.). All the mice received a test dose of organisms prepared from the same culture tubes of the strain Ty. 2, but one group received the bacilli suspended in mucin and the other the suspension in trypsin-broth without mucin. The test dose for each group was adjusted to contain between 10 and 100 average lethal doses ; for the mucin suspension this amount contained approximately 50,000 organisms, and for the trypsin-broth suspension, 75 million organisms. Groups of control mice that received normal rabbit serum were included in the experiment, and as is shown in the table, the two kinds of test suspension were titrated in these animals. Table I shows that a wide discrepancy in value is apparent, since 0·001 c.c. of the serum afforded almost complete protection against the test dose prepared in mucin, whereas the test dose in trypsin-broth required 0·1 c.c. of serum to give approximately the same degree of protection. This kind of result has been obtained regularly, and is independent of the particular Vi serum selected for the test ; it appears, therefore, to depend upon the widely different numbers of organisms in the test dose ; this explanation is considered later.

TABLE I.—*Illustrating the Protective Value of an Anti-Vi Serum.*

Test dose prepared in—	Number of organisms in test dose.	Serum.	I.V. dose of serum (c.c.).	Mice. Tested.	Mice. Survived.
Mucin	50,000	Vi	0·02	10	10
			0·005	10	10
			0·001	10	9
	50,000	Normal rabbit	0·02	10	0
	5,000			10	1
	500			10	3
Broth	75,000,000	Vi	0·10	10	9
			0·02	10	2
			0·005	10	0
	75,000,000	Normal rabbit	0·10	10	0
	7,500,000			10	6

The infecting doses were prepared from the strain Ty. 2. Agglutinin titres of Vi serum: Vi, 1 : 2000 ; O, < 1 : 100.

" Pure " O sera have also been tested by both methods. Table II summarizes the results of an experiment in which an O serum was tested against the fully virulent strain Ty. 2. The mice in both groups received approximately 10 average lethal doses, but whereas 0·001 c.c. of serum protected 7 of 10 mice in the group tested by the mucin method, 0·5 c.c. of serum failed to give more than a trace of protection to the mice which received the trypsin-broth suspension. There again, the only probable explanation of the wide discrepancy is the difference in the number of organisms used in the two methods of test. It is to be noted, however, that the difference in protective value obtained for the O antibody is much greater than that shown for the Vi antibody. This finding is clearly illustrated by the results of an experiment recorded in Table III, in which the rate of fall of protective value of a Vi

serum and of an O serum is compared when both were tested against increasing doses of organisms prepared from the virulent strain Ty. 2. Table III shows that when the number of organisms in the test dose is relatively small, as for example 50,000 (= 10 a.l.d.), the limit of efficiency for both sera is about the same, namely 0·001 c.c. As the number of organisms is increased, for example up to 5 million (= 1000 a.l.d.), the efficiency of the O antibody falls almost to zero, whereas that of the Vi antibody is reduced 50–100-fold.

TABLE II.—*Illustrating the Protective Value of an Anti-O Serum.*

Test dose prepared in—	Number of organisms in test dose.	Serum.	I.V. dose of serum (c.c.).	Mice. Tested.	Mice. Survived.
Mucin	50,000	O	0·02	10	10
			0·005	10	8
			0·001	10	5
	50,000	Normal rabbit	0·02	5	0
	5,000			5	1
Broth	75,000,000	O	0·50	10	1
			0·10	10	0
			0·02	10	0
	75,000,000	Normal rabbit	0·2	5	0
	7,500,000			5	4

The infecting doses were prepared from the strain Ty. 2. Agglutinin titres of O serum: O, 1 : 20,000; Vi, < 1 : 50.

TABLE III.—*A Comparison of the Protective Value of the Vi and the O Antibody Against Experimental Infection with a Fully Virulent (Vi + O) Strain (Ty. 2).*

Number of organisms in test dose.	Anti-Vi serum. I.V. dose (c.c.).	Anti-Vi serum. Mice. Tested.	Anti-Vi serum. Mice. Survived.	Anti-O serum. I.V. dose (c.c.).	Anti-O serum. Mice. Tested.	Anti-O serum. Mice. Survived.
50,000	·005	5	5	0·005	5	5
	·001	5	5	0·001	5	4
500,000	·02	5	5	0·10	5	3
	·005	5	5	0·02	5	2
	·001	5	3	0·005	5	0
5,000,000	·10	5	4	0·50	5	2
	·02	5	3	0·10	5	0
	·005	5	0	0·02	5	0

Agglutinin titres of Vi serum: Vi, 1 : 2,000; O, < 1 : 100.
" " O " : Vi, < 1 : 50; O, 1 : 20,000.

In a further series of protection experiments the infecting doses were prepared from relatively avirulent strains of *Bact. typhosum*. For this purpose an O-agglutinable strain (O 901) and a rough Vi strain (T. 6 S) were selected. These strains have already been investigated (Henderson and Morgan, 1938), and it has been found that the average lethal dose of either strain, when given

without mucin, contains very large numbers of organisms—approximately 500 millions. With the addition of mucin, however, the number of organisms required is reduced 100–1000-fold, and by this method the protective value of the O and the Vi antibody has been estimated. In the first place it has been found that a pure Vi serum fails completely to protect against 10 a.l.d. of the strain "O 901", and that a pure O serum is completely without influence on the course of infection with a similar number of average lethal doses of the rough Vi strain; these results are to be expected. In homologous protection tests, however, some interesting data were obtained. Table IV summarizes the results of one experiment, in which the protective value of a pure Vi serum was tested in one group of mice against infection with the rough Vi strain T. 6 S., and in another group of mice against infection with the fully virulent strain Ty. 2. Both test suspensions were prepared in mucin, and reference to the table will show that each was adjusted to contain approximately 10 a.l.d. For the avirulent strain this dose contained 50,000,000 organisms and for the virulent strain 50,000 organisms. It will be noted that 0·02 c.c. of the serum represents the limit of protective efficiency against infection with the avirulent strain, and that 0·001 c.c. protected approximately the same number of mice against infection with the virulent strain. Thus, approximately 20 times less serum is required to protect against a virulent strain than against an avirulent one. The key to this apparent paradox is to be found in the following results that are included in this experiment: A group of mice receiving the same sample of Vi serum was tested with approximately 10 a.l.d. of the fully virulent strain Ty. 2 suspended in trypsin-broth; the number of organisms present in this test dose is similar to that in the dose of avirulent organisms

TABLE IV.—*Illustrating the Protective Value of an Anti-Vi Serum Against Infection with a Rough Vi Strain (T. 6 S.) and a Vi + O Strain (Ty. 2).*

Test suspension prepared from—	Number of organisms in test dose.	Serum.	I.V. dose of serum (c.c.).	Mice. Tested.	Mice. Survived.
Rough Vi strain (T. 6 S.) in mucin	50,000,000	Vi	0·10	10	10
			0·02	10	4
			0·005	10	0
			0·001	10	0
	50,000,000	Normal rabbit	0·10	10	0
	5,000,000			10	2
Vi + O strain (Ty. 2) in mucin	50,000	Vi	0·005	5	5
			0·001	5	3
	50,000	Normal rabbit	0·005	5	0
	5,000			5	1
Vi + O strain (Ty. 2) in broth	75,000,000	Vi	0·10	10	8
			0·02	10	3
			0·005	10	0
	75,000,000	Normal rabbit	0·10	5	0
	7,500,000			5	3

Agglutinin titres of Vi serum: Vi, 1 : 1500; O, < 1 : 100.

(T. 6 S.) suspended in mucin. As a result of this adjustment the values obtained for the serum against infection with the virulent and avirulent strains are approximately the same, for in each case 0·02 c.c. represents the limit of efficiency of the serum.

Similar comparative tests have been carried out in which the protective value of a pure O serum has been estimated. The relatively avirulent strain 0901 and the Vi + O strain Ty. 2 were used as the test organisms. Table V summarizes the results of one of these experiments, and shows that approximately 10 a.l.d. of the strain O 901 required 0·02 c.c. of the O serum to give a satisfactory degree of protection, whereas 0·001 c.c. of the same serum gave a solid protection against 10 a.l.d. of the virulent strain Ty. 2: a result similar to that recorded in Table IV. Thus it is shown that an O serum is less effective against a given number of a.l.d. of an O strain than it is against infection with the same number of lethal doses of a fully virulent Vi + O strain.

TABLE V.—*Illustrating the Protective Value of an Anti-O Serum Against Infection with an O Strain* (O 901) *and a Vi + O Strain* (*Ty.* 2).

Test suspension prepared from—	Number of organisms in test dose.	Serum.	I.V. dose of serum (c.c.).	Mice. Tested.	Mice. Survived.
O strain (0901)	75,000,000	O	0·10	10	10
			0·02	10	8
			0·005	10	3
			0·001	10	1
	75,000,000	Normal rabbit	0·10	10	0
	7,500,000			10	6
Vi + O strain (Ty. 2)	50,000	O	0·005	5	5
			0·001	5	5
	50,000	Normal rabbit	0·005	5	0
	5,000			5	0

Agglutinin titres of O serum: O, 1 : 30,000; Vi, < 1 : 50.

From these and other experiments it appears that the protective efficiency of Vi or O antibody is related to the mass of antigen or bacterial substance introduced in the test dose of living organisms, rather than to the number of lethal doses contained therein or to the so-called virulence of the test strain. This hypothesis was finally tested by experiments in which killed bacterial bodies or extracted Vi or O substance were introduced into the body of the test animal after the administration of the immune-serum and immediately prior to the introduction of the test dose. In most of these experiments a pure Vi serum was tested, and the infecting suspension was prepared from the strain Ty. 2 and suspended in mucin. Extracts or killed suspensions of the rough Vi strain T. 6 S. were used as the additional antigen. Orientating experiments showed that the intraperitoneal route was unsatisfactory for the administration of the killed suspension or extract; with comparatively small doses of these antigens the protective value of the immune-serum remained unaltered, whereas with larger doses their direct toxic effect was such that the

a.l.d. of the living test suspension was lowered. The following method was finally adopted. Mice received the immune-serum as in previous tests; 18 hours later an alcohol-killed suspension of T. 6 S. or a Vi extract of the same strain was given intravenously, and immediately thereafter approximately 10 a.l.d. of the living suspension in mucin was injected intraperitoneally. Two groups of control mice which received normal rabbit serum were included in each experiment; both were given the same graded doses of the living suspension; one group received no further treatment, and the other was given the selected dose of additional antigen. By this means the influence of the antigenic substances injected intravenously on the lethal action of the living suspension was estimated. The results of one experiment conducted on these lines are summarized in Table VI. Each of three groups of mice received the same graded doses of serum. One group (A) had no treatment other than the injection of the test dose of living organisms; the second group (B) was given in addition an intravenous dose of 250 million alcohol-killed organisms (T. 6 S.); and the third group (C) received 1000 millions of the same organism intravenously. The deaths among mice in the normal serum controls show that the intravenous dose had no appreciable influence upon the a.l.d. The mice in the three groups were, therefore, tested with approximately 10 a.l.d. (50,000 organisms) of the living suspension. The influence of the additional killed suspension upon the protective value of the serum is clearly demonstrated, for whereas 0·001 c.c. of serum protected 100 per cent. of mice in

TABLE VI.—*Showing the Influence of Vi Antigen Injected Intravenously on the Protective Value of an Anti-Vi Serum.*

Dose of killed rough Vi suspension (T. 6 S.) given I.V.	Number of organisms in test dose of living suspension (Ty. 2).	Serum.	I.V. dose of serum (c.c.).	Mice. Tested.	Mice. Survived.
Nil (A)	50,000	Vi	0·02	5	5
			0·005	5	5
			0·001	5	5
	50,000	Normal rabbit	0·02	5	0
	500			5	3
250×10^6 organisms (B)	50,000	Vi	0·02	5	5
			0·005	5	5
			0·001	5	0
	50,000	Normal rabbit	0·02	5	0
	500			5	4
1000×10^6 organisms (C)	50,000	Vi	0·02	5	5
			0·005	5	0
			0·001	5	0
	Nil; mucin only	Vi	0·02	5	5
	50,000	Normal rabbit	0·02	5	0
	500			5	1
	Nil; mucin only	Normal rabbit	0·02	5	5

Agglutinin titres of Vi serum: Vi, 1 : 2000; O, < 1 : 100.

Group A, 0·005 c.c. was required in Group B and 0·02 c.c. in Group C. This result bears a close similarity to those obtained in the previous experiments. The inference to be drawn is that in the control of experimental infection of mice with *Bact. typhosum* the protective value of Vi or O antibody is determined by the number of organisms introduced in the test dose; the virulence of the test strain and the lethal power of the test dose are factors of only secondary importance.

DISCUSSION.

The earlier experiments of Grinnell (1932) and of Perry, Findlay and Bensted (1933 *a* and *b*) on the infection of mice with *Bact. typhosum* showed such regular results that other workers were encouraged to use this test in the assay of typhoid antigens and antibodies. Thus Felix and Pitt (1934) employed the method in their work on the detection and immunological properties of the Vi antigen. Their observations have been confirmed by Kauffmann (1935), Ørskov and Kauffmann (1936), Schütze (1936), Grasset and Lewin (1937) and many others. The mouse protection test has been continuously used for determining the biological relations of the different antigens.

Bact. typhosum is not a natural pathogen for the mouse, and this species is relatively insusceptible to the lethal action of the organism or its products. The experimental disease, therefore, differs markedly from an infection of the mouse with *Bact. aërtrycke*, or with certain strains of pneumococcus which, although seldom recorded as a natural pathogen for the mouse, may be characterized by a high degree of virulence for this animal. This difference is reflected in the tests with *Bact. typhosum* as normally carried out. Relatively enormous numbers of the bacilli suspended in broth or in saline are needed to establish a fatal infection although, as Ørskov and Kauffmann (1936) have shown, relatively small numbers of organisms, when injected into normal mice, can establish an infection which may persist for at least 14 days without producing clinical signs of illness. It can readily be shown that a lethal dose of killed organisms prepared from the most virulent strains of *Bact. typhosum* contains an amount of bacterial substance comparable to that which may be expected to accumulate by the multiplication, through only five or six generations, of a fatal dose of living organisms prepared from the same strain. This mode of infection, therefore, resembles in many respects a toxicity test, and the tests of active and passive protection which have been based on it may be likened to neutralization *in vivo* of toxin and antitoxin, rather than to a typical anti-invasive experiment (Ørskov and Kauffmann 1936.

The value of mucin for enhancing the virulence of *Bact. typhosum* when given intraperitoneally to mice was first reported by Rake (1935). Siler (1936), Buttle *et al.* (1937) and Henderson and Morgan (1938) have confirmed this finding, and have used mucin as an adjuvant to the test dose in their protection experiments. The mode of action of the mucin in enhancing virulence is still obscure, but the results reported in the present paper alone testify to the remarkable effect produced by it. Both virulent and relatively avirulent strains are influenced by its presence, and the number of organisms forming a lethal dose may be reduced 1000- to 10,000-fold. For example, if

the average lethal dose of a suspension in broth contains 5,000,000 organisms, the corresponding dose of the same suspension incorporated in mucin will contain about 500 organisms. As far as has been determined mucin is entirely without influence on the toxicity for mice of killed suspensions of *Bact. typhosum*, and strains grown in its presence on artificial culture-media appear to retain their antigenic composition unaltered. The disadvantages in its use may be two-fold. First, it is possible that only special preparations of gastric mucin of porcine origin are effective. The writer has used only the one (from Wilson Laboratories, Chicago) utilized by Miller (1935) and recommended by Rake (1935) for tests with *Bact. typhosum*. Secondly, a large dose of mucin when given intraperitoneally has been reported as having been toxic for the mouse ; in the writer's experience, however, 0·45 c.c. of a 5 per cent. emulsion in distilled water is completely non-toxic.

In earlier experiments (Henderson and Morgan, 1938) the mucin technique was used in the assay of the Vi and the O antibody. The results obtained did not agree with those recorded by Felix and others from protection tests in which the living bacteria were injected without mucin. The first experiments in the present paper show the difference in protective value observed when both methods are tested simultaneously, and if the protective value of Vi or O antibody is taken as an indicator of the sensitivity of the test it may seem that the mucin method is to be preferred.

The evidence afforded indicates that the protective value of Vi or O antibody is related to the number of bacteria in the test dose ; tests with relatively avirulent strains substantiated this view. If the term " virulence " is defined as the power of an organism to invade the tissues of the host and induce a fatal issue, it may reasonably be assumed that the more virulent the strain, the stronger will be the active or passive defence required to control the attack. However difficult it may be to determine whether this correlation exists in the pathogenesis of typhoid fever in man, the assumption is clearly inapplicable to the prophylaxis of the experimental disease in the mouse. In this animal the success of serum prophylaxis depends primarily upon the mass of bacterial antigen which may accumulate in its tissues ; the so-called virulence of the test strain is only of secondary importance in this respect. The factors which actually determine the virulence of any strain are not yet clearly defined ; the most virulent strains contain both the Vi and the O antigen, but do not necessarily contain either of these antigens in significantly greater amounts than avirulent strains. Further, the experiments reported in this paper as well as those of Ørskov and Kauffmann (1936) make it clear that strains of *Bact. typhosum* devoid of one or other of these antigens may readily multiply and remain alive in the mouse. It may be that the Vi and the O antigen by their combined action facilitate a rapid multiplication of organisms in the animal body and thereby give rise to an acute toxæmia. The comparatively small numbers of virulent organisms which are necessary to establish a fatal infection can be readily inactivated by a small prophylactic dose of Vi or O serum. On the other hand, in the control of an infection with a relatively avirulent strain, the large number of organisms introduced in the test dose and the massive accumulation of antigen necessitate a correspondingly large increase in the amount of antibody.

SUMMARY.

The efficiency of the Vi and the O antibody in the prophylaxis of experimental infection of the mouse with *Bact. typhosum* is determined by the mass of bacterial antigen which accumulates in its tissues.

The amount of either of these antibodies that is required to protect against infection with a fatal dose of living organisms is determined by the number of organisms in the dose and not by the virulence of the test strain. Thus the Vi or the O antibody is more efficient in the prophylaxis of infection with a small number of fatal doses prepared from a fully virulent strain than with the same number of fatal doses prepared from a relatively avirulent strain.

The specificity of the Vi and the O antibody can be clearly demonstrated by cross-protection tests against infection with a relatively avirulent rough Vi strain, or with an O-agglutinable strain which has none of the Vi antigen or only traces of it.

Although either the Vi or the O antibody will protect against infection with a moderate number of fatal doses of a fully virulent Vi + O strain, the Vi antibody is relatively more efficient than the O antibody against large multiples of a fatal dose prepared from this strain.

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EXPERIMENTS WITH THE Vi ANTIGEN OF *BACT. TYPHOSUM* AND WITH TWO NEW ANTIGENIC SUBSTANCES EXTRACTED FROM STRAINS OF THIS ORGANISM.

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THE extraction of strains of *Bact. typhosum* with anhydrous diethylene-glycol has been shown to yield substances that are antigenic and that are apparently free from protein (Henderson and Morgan, 1938). The choice of suitable strains for extraction made it appear that the Vi and the O antigen of *Bact. typhosum* exist as separate chemical entities. A preliminary study of the immunological properties of the extracted antigens indicated that the O substance is indistinguishable from the O antigen as it exists in the intact bacterial cell, but that the Vi antigenic complex is modified as a result of the extraction process. The immune-body produced by immunizing with Vi extract has been found to be much less effective in protecting mice against experimental infection than that which is produced by immunizing with suspensions of living Vi bacilli. A similar phenomenon was recorded by Felix and Bhatnagar (1935) with a Vi serum prepared by immunizing with a formolized extract. This experience indicates that Vi containing extracts retain the agglutinogenic and precipitinogenic characters of the intact cell, but are deficient in the property of stimulating the production of protective immune-body. In explanation of this anomaly two possibilities present themselves. Firstly, the untreated bacterial cell of *Bact. typhosum* may contain an antigen as yet undetected which is of importance in the production of protective antibody and which is insoluble in diethylene-glycol. An alternative is that the process of extraction causes certain groupings on the antigen molecule to undergo some chemical or physical modification. The experience of other workers in the isolation of bacterial antigens or their associated carbohydrate fractions lends support to this alternative, and the experiments reported in the present paper offer further evidence in its favour. During the course of this work it became clear that somatic antigens other than the Vi and the O substance are present in the intact cell of *Bact. typhosum*. These substances, which are apparently of a non-protein nature, have been extracted from suitable strains of *Bact. typhosum* with anhydrous diethylene-glycol and a preliminary study has been made of their immunological properties. The evidence suggests that these extracted substances may be similar to the R and ρ antigens described by Bruce-White (1933).

THE FUNCTIONAL DEFICIENCY OF THE EXTRACTED Vi ANTIGEN.

In a previous paper (Henderson and Morgan, 1938) the assumption was made that when a serum was prepared by immunizing rabbits with a suspension of living rough Vi bacilli, its protective value was reflected by the content of Vi agglutinin. On the basis of this assumption, the functional deficiency of a serum prepared by immunizing with an extract from a rough Vi strain was observed by comparing it in protection experiments with a serum of the same agglutinin titre prepared against living organisms. From these experiments it was concluded that there is at least a 20-fold decrease in the efficiency of the antibody produced by immunizing rabbits with Vi-containing extracts. To test the accuracy of this finding various sera have now been examined by agglutination, precipitation and complement-fixation tests, and by tests for their protective value against infection with homologous and heterologous strains. The earlier protection experiments were carried out with test doses of a fully virulent Vi + O strain. In view of the findings reported in a recent paper (Henderson, 1938) concerning the influence of the virulence of the test strain upon the protective action of the Vi and the O antibody, it was decided to test the deficient type of " pure " Vi serum against infection with a relatively avirulent rough Vi strain; for this purpose the strain " T. 6 S." was selected. Homologous sera prepared against the living organisms and the corresponding Vi extract were examined simultaneously; the mucin technique already described was used (Henderson, 1938). Table I summarizes the results of one experiment. The results of titrating the infecting suspension in mice which had received a dose of normal rabbit serum show that the test dose given to all mice treated with immune serum contained between 10 and 100 average lethal doses (a.l.d.) of the living organisms. The two " pure " Vi sera had the same agglutinin titre, but the serum prepared by immunizing with living organisms proved to be approximately 25 times more efficient in protection than the other sample. These sera had already been tested against infection with a heterologous and fully virulent Vi + O strain (Ty. 2), and the difference

TABLE I.—*A Comparison of the Protective Value of Two Types of Anti-Vi Serum.*

Antigen used for immunization.	Rabbit-serum. Agglutinin titre. Vi.	Rabbit-serum. Agglutinin titre. O.	I.V. dose (c.c.).	Mice. Tested.	Mice. Survived.	Number of organisms in dose.
Vi (living organisms)	2000	<1 : 50	0·1	10	10	50 × 10^6.
			0·02	10	4	
			0·005	10	0	
Vi (extract)	2000	1 : 50	0·5	10	6	50 × 10^6.
			0·1	10	0	
			0·02	10	0	
Nil; normal rabbit serum control	<1 : 10	<1 : 50		10	0	50 × 10^6.
			0·2	10	1	50 × 10^5.
				10	6	50 × 10^4.

The infecting doses were prepared from the relatively avirulent "rough" Vi strain T. 6 S.

in their protective values was shown to be of the same order as that recorded in Table I. It appears, therefore, that the virulence of the test strain has little or no influence upon the range of the discrepancy between the protective values of the two types of serum. For reasons previously given, however, both sera were about 20 times more efficient against infection with the fully virulent strain than they were against the avirulent one (Henderson, 1938).

Felix and Bhatnagar (1935) obtained a modified Vi antigen by extracting suitable strains of *Bact. typhosum* with 0·25 per cent. formol-saline. They also stated that a deficient Vi serum was produced by immunizing rabbits with a formolized suspension of the organisms. Neither the experiments of Felix and Bhatnagar, nor those of Henderson and Morgan (1938), gave conclusive evidence to show that the action of the formalin or the extraction process was directed in particular against the molecule of the Vi antigen; the possible presence of another labile somatic antigen had not been excluded. To investigate this point various absorption experiments were carried out. "Pure" Vi sera prepared by immunizing with organisms or Vi extracts were absorbed with a formolized suspension or with a diethylene-glycol extract. Complete absorption of the Vi agglutinin was readily accomplished by the use of the formolized suspension; the method of optimal proportion was used for the precipitation of Vi antibody with extracts, but complete removal of agglutinins was found to be difficult. The absorbed sera were tested by agglutination, and their protective value was estimated in mice against infection with a fully virulent strain. The results of one experiment are summarized in Table II. In this test a Vi serum prepared against living organisms of the strain T. 6 S. was absorbed with a formolized suspension of the homologous strain. The absorbing antigen was prepared from an 18-hour agar-culture of the strain T. 6 S. The growth was suspended in 0·25 per cent. formol-saline and washed once in this diluent. The suspension was then standardized by an opacity method to contain 2000 million organisms per c.c. and stored for two days at room temperature and about three weeks at $\pm$ 1° C. Before use the suspension was centrifuged and twice washed in saline. The technique for absorption and the scheme of the protection test have already been described (Henderson and Morgan, 1938; Henderson, 1938). Absorption of another portion of the same serum with a formolized suspension of an O strain (O 901) was carried out as a control, and Table II shows the *in vitro* and *in vivo* results obtained with the untreated serum and the differently absorbed portions of it. It is clear that the formolized O suspension failed to remove any trace of Vi agglutinin or to alter the protective value, whereas the similarly treated Vi suspension removed the Vi agglutinin, and at the same time completely exhausted the protective antibody. If the treatment with formalin had destroyed a hypothetical but important labile antigen which was present in the original Vi suspension this effect might have been indicated by the appearance of some residual protective antibody in the serum absorbed with the formolized suspension. It is possible, however, that the treatment with formalin may have destroyed the antigenic properties without affecting the absorbing capacity of the hypothetical substance, and the same result may be expected to occur in extracts obtained by diethylene-glycol. The experiments described later in this paper prove the existence of other somatic antigens of

Bact. typhosum, but there is no indication that the action of formalin or the extraction process renders these substances non-antigenic. It appears, therefore, that the action of formalin or of the extraction process on the Vi antigen is to modify its molecular structure in such a way as to lead to the production, when used for immunization, of an "incomplete" antibody molecule.

TABLE II.—*The Influence of Absorption with Formolized Organisms on the Protective Value of an Anti-Vi Serum.*

Anti-Vi rabbit-serum.					
Antigen used for absorption.	Vi-agglutinin titre.	I.V. dose (c.c.).	Mice. Tested.	Mice. Survived.	Number of organisms in dose.
Unabsorbed	1 : 1500	0·005	10	10	50,000
		0·001	10	4	
Formolized O suspension	1 : 1500	0·005	10	9	
		0·001	10	6	
Formolized Vi suspension	1 : 20	0·5	10	0	
		0·2	10	0	
Normal rabbit serum control	<1 : 10	0·2	10	1	50,000
			10	0	5,000
			10	8	500

The infecting doses were prepared from the virulent strain Ty. 2.

Further evidence on this point was obtained from an examination of the complement-fixation reaction of "pure" Vi serum when in contact with Vi antigen, either as it is present in the intact cell or in partially purified diethylene-glycol extracts. For this complement-fixation test a standard dose of amboceptor (5 M.H.D.) was used together with a varying number of M.H.D. of guinea-pig complement. The antigens were tested immediately before use against 2 M.H.D. of complement; the concentration of antigen used in the test proper was one-fourth of the smallest amount which inhibited lysis. The test was conducted in a total volume of 1·5 c.c. The mixture of serum, complement and antigen was incubated for one hour at 37° C., and thereafter 0·5 c.c. of a 3 per cent. suspension of sheep red cells was added. Readings were taken after incubation for one hour at 37° C., and again after standing overnight at ± 1° C. In all cases non-specific serum controls were included in the test. Table III gives the results of one experiment in which various Vi sera were compared in cross complement-fixation tests with two types of Vi antigen, one antigen consisting of "rough" Vi organisms and the other of partially purified Vi extract. The organisms were obtained from an 18-hour agar-culture of the strain T. 6 S. The growth was washed off in saline, centrifuged and rapidly dried in acetone as previously described (Henderson and Morgan, 1938); immediately before use the dried substance was suspended in saline, washed four times and finally resuspended to the required opacity in the same diluent. The extracted Vi antigen had been prepared from the same strain (T. 6 S.) by the diethylene-glycol process and was partially purified by precipitation with 30–50 per cent. of acetone; immediately before use a known amount of the dried substance was suspended in saline. Table III shows that the "rough"

TABLE III.—*Complement-fixation Test with Two Types of Vi Serum.*

Antigen used in the test.	Rabbit serum. Antigen used for immunization.	Identification number.	Serum dilution. 1 : 50.	1 : 100.	1 : 200.	1 : 500.	1 : 1000.	1 : 2000	Agglutinin titres of serum. Vi.	O.	H.
Rough Vi organisms	Rough Vi organisms	24	++++	+++±	±	(±)	−	−	1 : 1000	1 : 50	1 : 2000
		58	++++	++++	++++	+++±	+±	(±)	1 : 4000	<1 : 50	1 : 10,000
		63	++++	++++	+++±	++±	±	−	1 : 2000	<1 : 50	1 : 5000
	Rough Vi extract	16	±	(±)	−	−	−	−	1 : 1000	1 : 50	1 : 1000
		71	(±)	−	−	−	−	−	1 : 2000	1 : 200	<1 : 50
		79	(±)	−	−	−	−	−	1 : 2000	1 : 50	<1 : 50
Rough Vi extract	Rough Vi organisms	24	++++	++++	+++±	(±)	−	−		..	
		58	++++	++++	++++	++++	++±	(±)		..	
		63	++++	++++	++++	++	(±)	−		..	
	Rough Vi extract	16	++++	++++	++++	+++±	±	−		..	
		71	++++	++++	++++	++++	+++	(±)		..	
		79	++++	++++	++++	++++	++±	(±)		..	

Test conducted in the presence of 2·5 M.H.D. of guinea-pig complement. ++++ to (±) = degrees of inhibition of lysis of sheep red cells (++++ = complete inhibition).

Vi organisms gave a positive result with the sera prepared by immunizing with living organisms, but no complement-fixation occurred in the presence of sera prepared against extracted Vi antigen—a result which agrees with those obtained in passive protection tests. On the other hand, it appears that when Vi extract is used as antigen strong complement-fixation occurs with both types of serum. This latter finding, which at first sight is difficult to explain, is analysed in the account of subsequent experiments.

TWO NEW ANTIGENIC SUBSTANCES EXTRACTED FROM *BACT. TYPHOSUM*.

In the course of the present experiments it was found necessary to examine several strains of *Bact. typhosum* which were regarded as being devoid of both the Vi and the O antigen. Two strains (R. [Ty. 2], M.S.R.), believed to satisfy this requirement, were kindly supplied by Dr. Felix, and a study of their immunological properties has led to the finding of two new somatic antigens which are apparently free from protein. The lack in these strains of O or Vi antigen was first of all tested by immunizing rabbits with a living suspension of the organisms. For each strain six animals were used, and after four courses of immunization no significant rise in Vi or O agglutinin was detected in any of the sera. Both strains, however, are rich in H antigen, and the immune sera had H agglutinin titres ranging from 1 : 20,000 to 1 : 100,000.

Two dried preparations of each strain were prepared, and from them partially purified diethylene-glycol extracts were obtained by the methods described (Henderson and Morgan, 1938). The yield of extracted substance in each case appeared to be much less than that obtained from O or Vi strains of *Bact. typhosum*, and in the process of partial purification with acetone it was found that at least two-thirds by volume of this reagent was required to precipitate the extracted substance. The extracts could generally be obtained as white amorphous solids by rapid evaporation from a frozen aqueous solution, at a low temperature and *in vacuo* ; the dried material readily redispersed in water to form a stable suspension, but occasionally after dissolving in saline a small deposit settled out.

In a preliminary examination of the chemical properties of these extracts attention was confined to a repetition of the qualitative tests which were applied in the study of Vi and O extracts (Henderson and Morgan, 1938). The reaction of each " rough " extract in the presence of various reagents was found to be closely aligned to that of the O extracts. The biuret test, however, which is generally positive with O substance, was either negative or showed only a faint trace of reaction. Protein appeared to be absent from all the preparations; no precipitation occurred with trichloracetic acid, Esbach's reagent or salicylsulphonic acid. It is hoped to continue this work in collaboration with Dr. W. T. J. Morgan, when a more detailed investigation of the chemical properties of these substances will be undertaken.

The toxicity for mice of the " rough " fractions was found to be of the same low order as that recorded for Vi extracts. Mice of 18–22 g. were refractory to 2 mg. of the rough extract given intravenously, and a dose of 5 mg. killed only a small proportion of the animals.

The antigenic activity of each extract was tested in a group of 4 to 6 rabbits.

A sample of normal serum was first taken, and the animals were selected for immunization whose serum had an O-agglutinin titre of less than 1 : 50. The test material was given intravenously in from 4 to 6 doses ranging from 0·5 to 2·0 mg. ; each dose was separated by an interval of 2 or 3 days. The animals were bled 6 or 7 days after the last dose of antigen. Each rabbit received from two to four courses of immunization on this plan.

The immune sera prepared in this way were examined in agglutination tests for the presence of Vi and O antibody with the strains " Watson " and " O 901 " respectively. Whereas the living " rough " suspensions failed to stimulate the production of antibody which reacted with either of these strains, the sera prepared against the extracts were less regular in this respect. Occasional rabbits in any group produced sera which agglutinated to titres of 1 : 50 with the strain " Watson ", and one serum was found which agglutinated with the strain " O 901 " to a titre of 1 : 500. These values, however, were obtained only after prolonged immunization—generally after a fourth course. On the other hand, the serum from certain animals in the same group remained free from Vi or O agglutinin. It is not clear whether this production of antibody was, in fact, due to the presence of a trace of Vi or O substance which became concentrated in the extract, or whether an antibody to the " rough " substance exerted its influence in agglutination in a manner similar to that of O antibody in the presence of Vi + O strains. On the latter supposition the resistance to the action of O antibody is only relative ; a high concentration of this type of serum agglutininates even the most resistant strains. It has previously been reported that H antigen can be detected in diethylene-glycol extracts obtained from strains rich in flagella. This is probably due to " soluble " flagellar substance which passes the bacterial filters used in the preparation of the extracts. It was not surprising, therefore, to find that all the sera prepared against the " rough " extracts had H agglutinin titres ranging from 1 : 500 to 1 : 2000.

The extreme salt sensitiveness of the " rough " strains R. Ty. 2 and M.S.R. made agglutination tests impossible with living suspensions of these strains. I have found that even alcohol-treated suspensions used in a low concentration of saline are unsatisfactory for delicate tests, although by this means a general impression is gained concerning the reactivity of the various immune-sera. Thus, antibody to a somatic antigen of the rough living organisms, which was distinct from Vi or O antibody, was regularly produced by immunizing with the living rough suspensions. On the other hand, sera prepared against the rough extracts appeared to have little or none of this type of antibody. The indication was that if the extracts contained this " rough " substance, it was present either as a typical hopten or as a " modified " antigen. The precipitation and complement-fixation tests described below were designed to investigate this problem.

The partially purified " rough " extracts were examined in precipitation tests with the " rough " sera and with so-called " pure " O and " pure " Vi sera. Table IV summarizes the results of an experiment in which one " rough " extract from the strain " R. Ty. 2 " was tested. The table shows that a strong precipitation reaction was obtained with the " rough ", O, and Vi sera which had been prepared by immunizing with their respective extract. The

precipitation, however, was much weaker with serum prepared against the living organisms of the homologous " rough " strain, and only traces of precipitation occurred with O and Vi serum similarly prepared. The O, Vi and H agglutinin content of the various sera is also shown in Table IV, and it is clear that the positive precipitation results were independent of any of these antibodies. The test, therefore, indicates the presence of an antigen in the " rough " extract which is separate from O or Vi substance ; from the qualitative test described above it appears that this " rough " substance is of a non-protein nature. The weak precipitation reaction with sera prepared against the living organisms is not easily explained ; modifications of the antigen molecule as a result of the extraction process may be responsible, since the subsequent complement-fixation experiments demonstrate that an alteration in the "rough" antigen occurs as a result of the extraction.

TABLE IV.—*Precipitation Test with "Rough" Extract.*

Rabbit serum (0·1 c.c. per tube).	Rough extract (mg. per tube).				Agglutinin titres of serum.		
	0·5.	0·1.	0·02.	0·005.	Vi.	O.	H.
Rough (L)	++	+	(±)	−	? 1 : 5	1 : 50	1 : 10,000
Rough (E)	+++	+++	+++	+±	1 : 20	1 : 100	1 : 5000
Vi (L)	(±)	(±)	−	−	1 : 2000	<1 : 50	1 : 5000
Vi (E)	+++	+++	+++	++	1 : 2000	1 : 50	<1 : 50
O (L)	−	−	−	−	1 : 50	1 : 20,000	<1 : 50
O (E)	+++	+++	+	±	1 : 10	1 : 20,000	<1 : 50
Normal rabbit	−	−	−	−	<1 : 5	1 : 50	<1 : 50

(L) Refers to serum prepared by immunizing with living organisms. (E) Refers to serum prepared by immunizing with extracts. Total volume per tube adjusted to 1·0 c.c. with 0·85 per cent. NaCL. Readings taken after 24 hours (1 hour at 37° C. and then at ± 1° C.). +++ to ± = degrees of precipitation observed with the naked eye. (±) = trace recorded with the hand lens.

In a previous paper (Henderson and Morgan, 1938) it was stated that the specificity of the O and Vi extract was demonstrated by precipitation tests. The information now available shows that the O or Vi serum prepared by immunizing with the respective extract contains " rough " antibody which is common to both sera. It is clear, therefore, that neither O nor Vi extract is a single antigenic substance, since both contain a common " rough " antigen. It may be assumed that the absence of cross-precipitation between these antigens and the corresponding sera is due to the small amount of the "rough" antigen present in the Vi or the O extract. This belief is supported by details concerning the preparation of the three antigens. Thus, in the process of partial purification 30 to 50 per cent. by volume of acetone is used to precipitate the O or the Vi substance, whereas the " rough " antigen requires at least 60 per cent. by volume of acetone before a satisfactory precipitation is obtained.

In the light of these experiments a study was made of the complement-fixation reaction of the Vi, O, and " rough " antigen in the presence of the corresponding serum ; the method of test has been described above. Direct and cross complement-fixation tests as well as absorption experiments were carried out. The experiment summarized in Table V has been chosen primarily to

demonstrate that the "rough" antigen and antibody are common to all strains of *Bact. typhosum*. For this experiment two types of "rough" antigen were used, one consisting of "rough" organisms and the other of "rough" extract. Both were prepared from the strain R. Ty. 2 in a manner similar to those antigens which were used in the test summarized in Table III. A Vi, an O and a "rough" serum prepared by immunizing rabbits with living suspensions of the respective strains were tested together with similar type sera prepared against partially purified diethylene-glycol extracts of these organisms. The normal serum from each animal providing immune-serum was included in the test, as well as various heterologous immune-sera. These controls, which are omitted from Table V, showed no trace of complement-fixation with either antigen. The Vi, O and H agglutinin-content of the sera is recorded in the table, and it appears that the complement-fixing power of a serum in the presence of either of the "rough" antigens is independent of any of these antibodies. Evidence for the presence of antibody to a third type of somatic antigen is, therefore, clearly established. The complement-fixing power in the presence of "rough" organisms is reminiscent of that which is obtained with Vi sera and organisms in the experiment recorded in Table III; thus, a serum prepared against the living "rough" organisms gives a strong positive reaction with the homologous antigen, whereas a negative result is obtained with the organisms in the presence of a serum prepared against a "rough" extract. Table V also shows that the same kind of result is obtained with the two types of Vi serum tested in the presence of "rough" organisms, and although to a lesser extent, this is also true for the O sera. It appears, therefore, that "rough" antibody is regularly produced in animals immunized with living suspensions of any variant of *Bact. typhosum*. The "rough" antigen is, however, so modified by the process of extraction that the antibody produced no longer reacts in complement-fixation tests with the antigen of the intact cell. An unexpected result of this experiment is the positive complement-fixation shown by all but one of the sera in the presence of the extracted "rough" substance. Precipitation tests with this antigen and the various antibodies had discriminated between sera prepared by immunizing with extracts and those prepared against living organisms; it is unusual to find an antigen which functions in complement-fixation, but fails to do so in precipitation tests.

Precipitation tests had failed to detect the presence of "rough" antigen in Vi or O extract, although sera prepared against these extracts have now been shown to possess "rough" antibody which reacts in precipitation tests with extracted "rough" substance. This "rough" component of Vi or O extract can be readily detected by complement-fixation tests. Table VI summarizes the results of one experiment which illustrates this point. The test was conducted on similar lines to the one recorded in Table V; the same sera were used and Vi antigens were substituted for the "rough" bacterial substance and extract. Reference to Table VI will show that there is conclusive evidence for the presence of "rough" antigen in the Vi extract. From similar experiments with O antigen one result deserves comment. It has been found that this substance suffers no modification during the process of extraction such as has been observed with Vi antigen. Thus the O antibody to extracted O antigen reacts strongly in complement-fixation tests with O

TABLE V.—*Complement-fixation-Test with Two Forms of "Rough" Antigen.*

Antigen used in the test.	Rabbit serum: Antigen used for immunization.	Serum dilution. 1:50.	1:100.	1:200.	1:500.	1:1000.	1:2000.	1:5000.	1:10,000.	1:20,000.	Agglutinin titre of serum. Vi.	O.	H.
Rough extract	Rough (L)	++++	++++	++++	++++	++++	+++	±	—	—	? 5	50	10,000
	Rough (E)	++++	++++	++++	++++	++++	++++	+++±	++	—	20	100	5000
	Vi (L)	++++	++++	++++	+++±	—	—	—	—	—	2000	<50	5000
	Vi (E)	++++	++++	++++	++++	++++	++++	(±)	—	—	2000	50	<50
	O (L)	+++±	+++	+	—	—	—	—	—	—	50	20,000	<50
	O (E)	++++	++++	++++	++++	++++	+++±	—	—	—	10	20,000	<50
Rough organisms	Rough (L)	++++	++++	++++	++++	++++	+++±	±	—	—		..	
	Rough (E)	±	—	—	—	—	—	—	—	—		..	
	Vi (L)	++++	++++	++++	+++	±	—	—	—	—		..	
	Vi (E)	++	±	—	—	—	—	—	—	—		..	
	O (L)	+++	++	±	—	—	—	—	—	—		..	
	O (E)	+±	(±)	—	—	—	—	—	—	—		..	

(L) = Serum prepared by immunizing with living organisms. (E) = Serum prepared by immunizing with extracts. Test conducted in the presence of 2·5 M.H.D. of guinea-pig complement.

TABLE VI.—*Complement-fixation Test with Two Forms of Vi Antigen.*

Antigen used in the test.	Rabbit serum: Antigen used for immunization.	Serum dilution: 1:50.	1:100.	1:200.	1:500.	1:1000.	1:2000.	1:5000.	Agglutinin titre of serum. Vi.	O.	H.
Vi extract	Vi (L)	++++	++++	++++	+++	(±)	—	—	2000	<50	5000
	Vi (E)	++++	++++	++++	++++	+++±	±	—	2000	50	<50
	Rough (L)	++++	++++	++++	++++	+++±	++±	±	?5	50	10,000
	Rough (E)	++++	++++	++++	++++	++++	+++	±	20	100	5000
	O (L)	++±	(±)	(±)	—	—	—	—	50	20,000	<50
	O (E)	++++	++++	++++	+++±	+	—	—	10	20,000	<50
Vi organisms	Vi (L)	++++	++++	+++±	++	(±)	—	—		..	
	Vi (E)	—	—	—	—	—	—	—		..	
	Rough (L)	++++	++++	++++	++±	(±)	—	—		..	
	Rough (E)	(±)	—	—	—	—	—	—		..	
	O (L)	+±	+	—	—	—	—	—		..	
	O (E)	—	—	—	—	—	—	—		..	

(L) = Serum prepared by immunizing with living organisms. (E) = Serum prepared by immunizing with extracts. Test conducted in the presence of 2·5 M.H.D. of guinea-pig complement.

organisms. This result is in agreement with that obtained in passive-protection experiments (Henderson and Morgan, 1938), in which the O antibody to extracted substance was as effective as that produced by immunizing with living organisms.

The results of the direct and cross complement-fixation tests have been verified and extended by absorption experiments. Table VII summarizes the essential features of one experiment in which various sera were absorbed with " rough " organisms of the strain R. Ty. 2. The absorbed and unabsorbed portions of the sera were then tested in agglutination and complement-fixation with the necessary antigens. The results of this experiment may be summarized as follows :

(1) " Rough " antibody produced by the " rough " antigen of the intact cell is removed, by absorption with the " rough " organisms, from Vi or " rough " serum prepared against living organisms.

(2) Vi antibody produced by the Vi antigen of the intact cell is not affected by the absorption with the " rough " organisms.

(3) The complement-fixing titre of " rough " antibody prepared by immunizing with the " rough " or the Vi extract remains unchanged after absorption with " rough " organisms.

The first and second statement in this summary confirm the earlier findings. The third statement, however, requires some further explanation. It has already been shown that " rough " antibody produced by immunizing with extracted antigen fails to fix complement in the presence of " rough " organisms. On the other hand, antibody to the " rough " organisms fixes complement in the presence of " rough " extracted antigen as well as of the homologous antigen. An explanation of the latter result is found by postulating the existence of yet another antigen of *Bact. typhosum* ; one which is soluble in diethylene-glycol is of a non-protein nature, and is not structurally altered as a result of the extraction process. The position of this hypothetical substance in the intact cell may be such that it cannot take an active part as a receptor in the *in vitro* tests with the organisms although it may still act as an antigen *in vivo*. The absorption experiment reported in Table VII offers evidence in support of this suggestion. The results indicate that there are at least two fractions in the extracted " rough " substance which are concerned in complement-fixation. The first of these is responsible for the production of a specific complement-fixing antibody ; it is probably the substance which, in the intact cell, remains passive in *in vitro* tests. The second fraction when it exists as a constituent of an extract appears to be incapable of stimulating the production of complement-fixing antibody, although it retains the power of fixing complement in the presence of antibody produced by immunizing with the whole organisms ; this substance is probably the equivalent of the dominant surface antigen of the " rough " organism. It is not yet definitely known whether the modification which this second fraction undergoes during the process of extraction reduces it to a true hapten form or whether it still retains certain antigenic properties. That the latter alternative is the probable one is indicated by the results of certain absorption experiments. For example, it has been shown that a living suspension of " rough " organisms will remove in part the precipitins from a serum prepared against " rough " extracts. The other reactions

of the " rough " organisms in the presence of this type of serum that have been described above give reasonable grounds for concluding that the precipitating antibody absorbed by the organisms is produced by the second fraction of the extract.

TABLE VII.—*Complement-fixation Test with Absorbed Sera.*

Rabbit-serum.		Titre of complement-fixation with antigens			
Antigen used for immunization.	Absorbed in dilution 1 : 25 with bacilli from strains :	Rough organisms.	Rough extract.	Vi organisms.	Vi extract.
Rough organisms	Rough	50	50	50	50
	Vi	50	50	50	50
	Unabsorbed	5000	10,000	2000	5000
Rough extract	Rough	50	5000	0	5000
	Vi	50	5000	0	5000
	Unabsorbed	50	5000	0	5000
Vi organisms	Rough	0	0	500	0
	Vi	0	0	0	0
	Unabsorbed	500	1000	1000	1000
Vi extract	Rough	0	2000	0	2000
	Vi	0	2000	0	2000
	Unabsorbed	0	2000	0	2000

Titre 0 = negative result in a dilution of 1 : 50. Test conducted in the presence of 2·5 M.H.D. guinea-pig complement.

The reasoning which has been applied in the explanation of the preceding results is applicable also to experiments with Vi antigen. Thus Vi extract will fix complement in the presence of homologous serum or serum prepared against living Vi organisms, as well as with the antibody obtained by immunizing with " rough " organisms. On the other hand, " rough " Vi organisms will fix complement only in the presence of homologous serum or serum produced against " rough " organisms. Here, also, is evidence for the presence in " rough " Vi strains of two somatic antigens other than Vi substance.

The *rôle* that these two new antigens may play in infection with *Bact. typhosum* and in the production of protective antibodies remains to be determined.

DISCUSSION.

The experiments on Vi antigen that are reported in this paper confirm the earlier accounts concerning the lability of the Vi antigenic complex as it exists in the intact bacterial cell. The essentially simple process of extracting the dried organisms with an anhydrous organic solvent at a neutral pH appears sufficient to reduce the extracted substance to a position intermediate between that of the complete antigen of the intact cell and a true hapten. The change that occurs seems to affect that portion of the antigenic complex which stimulates the production of protective antibody. Thus it has been estimated that the prophylactic efficiency of this type of serum is about 25 times less than

one prepared by immunizing with the living organisms. The difference in value exhibited by the two types of serum was judged from knowledge of the Vi agglutinin content; the experience of Felix and his co-workers as well as my own have justified the conclusion that the protective efficiency of a Vi serum prepared against living organisms was correlated with the content of this agglutinin. Up to the present, however, there was no clear evidence that the extraction process impaired the immunizing efficiency of the Vi antigen molecule in particular; the presence of an unknown labile somatic component had not been excluded, and it was possible that such a hypothetical substance was insoluble in diethylene-glycol. The absorption experiments and complement-fixation tests described in the present paper were designed to investigate this problem, and although new somatic antigens of *Bact. typhosum* have been discovered, strong support is lent to the view that the Vi antigen molecule is, in fact, modified as a result of the extraction process. One portion of the molecule which suffers structural change is that which is concerned in the production of complement-fixing antibody, since serum prepared by immunizing with Vi extract does not fix complement in the presence of Vi organisms. It is interesting to observe, however, that this type of serum, though devoid of complement-fixing antibody for the whole organisms, still retains a proportion of its protective efficiency. It may be concluded, therefore, that the groupings on the antibody molecule which play an active part in protection tests are not necessarily identical with those concerned in the fixation of complement.

While pursuing the inquiry into the structural alterations in Vi antigen, evidence for the presence of two other somatic antigens of *Bact. typhosum* was obtained. These substances have been found to exist as chemical entities which are separate and distinct from the Vi and the O antigen. The evidence obtained indicates that these substances may be similar to, if not identical with the R and ρ antigens described by Bruce White (1233). Thus one of them probably represents the surface antigen of a typical " rough " form of *Bact. typhosum*, and there is evidence to believe that the other lies in the deeper layers of the bacterial substance. The extraction of suitable strains with, diethylene-glycol, according to Morgan's (1937) original method, yields this second substance in an actively antigenic state, whereas the " rough " surface antigen undergoes structural modification similar perhaps to that of the Vi antigen. Partially purified extracts of the " rough " organisms contain both these new substances in a form apparently free from protein. A method for separating them with the object of subjecting them to chemical analysis has not yet been devised. A preliminary chemical examination of some of the extracts containing both these components, however, indicates that they may be more closely related to the O than the Vi substance of *Bact. typhosum*. That they are of the nature of group antigens has been clearly demonstrated in precipitation and complement-fixation tests with Vi, O and " rough " sera; this finding raises the problem of the further chemical purification of the Vi and the O antigen in extracts obtained by the diethylene-glycol process.

The part played by these two new antigens in infection with *Bact. typhosum* is not yet known. Their presence in typical " rough " strains and the low order of toxicity manifested by extracts containing them indicates that they take no important share in the lethal action of the typhoid bacillus.

The method of extracting bacterial antigens with anhydrous diethylene-glycol which was introduced by Morgan (1937) offers wide possibilities not only as a means of extracting the so-called specific bacterial antigens, but as a method which will help in the further analysis of the antigenic mosaic within the cell. It is unfortunate that as a result of this process certain of the antigens of *Bact. typhosum* suffer some structural modification, but this fact may in itself lead finally to a better knowledge of the factors which govern the specificity of the antigen-antibody reactions. For example, it would be of interest to know what changes, if any, the antigens undergo as a result of extraction from the cell either with trichloracetic acid according to Boivin's method or by the process of tryptic digestion which has been employed by Raistrick and Topley ; a comparison of the properties of the antigen so obtained with those of the diethylene-glycol extracts may add considerably to our knowledge concerning antigen structure and function.

SUMMARY.

A study has been made of some of the immunological properties of the substances obtained by extracting suitable strains of *Bact. typhosum* with anhydrous diethylene-glycol.

The earlier account of the functional deficiency of extracts containing Vi antigen has been confirmed. There is now evidence for believing that the portion of the Vi antigen molecule which is concerned with the production of complement-fixing antibody is structurally altered. The groupings on the antigen molecule that stimulate the production of protective antibody are not necessarily identical with those that are concerned in the production of complement-fixing antibody.

The extraction of suitable " rough " strains of *Bact. typhosum* with diethylene-glycol yields two antigenic substances, which are apparently free from protein. These antigens appear to be separate and distinct from the Vi or the O antigen. One of them probably represents the surface antigen of a typical " rough " strain of *Bact. typhosum* ; in the process of extraction it undergoes structural modification. The source of the other antigen may be the deeper layers of the bacterial substance ; it is probably unaltered by the process of extraction. Both of these new substances have been found in extracts prepared from so-called " pure " " rough " Vi strains as well as from " pure " O strains, and they probably represent constituents of the normal bacterial cell.

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THE EFFECT OF GASTRIC MUCIN ON THE PATHOGENICITY OF THE MENINGOCOCCUS AND OTHER ORGANISMS: WITH PARTICULAR REFERENCE TO ITS FRACTIONATION.

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GASTRIC mucin was first used experimentally for promoting bacterial infection in an attempt to reproduce gonococcal lesions in laboratory animals (Miller, 1933). It was assumed that since this organism invades the body naturally *via* a mucous surface, " mucin " might be of value in favouring the entry and spread of the gonococcus under experimental conditions. During this investigation it was found that mucin could also be used for promoting meningococcal infections in mice (Miller and Castles, 1936), animals which hitherto had proved resistant to the organism. In 1932 Nungester *et al.* noted that hæmolytic streptococci, *S. aureus* and the pneumococcus all had their pathogenicity for mice exalted, if the animals were injected intraperitoneally with a suspension of the organisms in 4% gastric mucin; while in 1936 they confirmed the effect on the streptococcus and pneumococcus, but were unable to obtain similar results with staphylococci, both recent and old strains. In 1937, Chandler *et al.* and Fothergill *et al.* amply proved the power of mucin to enhance the pathogenicity of *H. influenzæ* towards mice.

With reference to other substances which might produce a similar effect, agar, starch and gelatin have been tested (Miller and Castles, 1936), and found to have a similar though much weaker action, while peptone, indian ink, nutrient agar, testicular extract, etc. (Nungester *et al.*, 1936), have little or no comparable effect.

THE EFFECT OF MUCIN UPON THE PATHOGENICITY OF THE MENINGOCOCCUS.

Preparation of Mucin Suspension or Solution.

A plain 5 per cent. suspension of mucin in normal saline was first used. After boiling for 20 minutes this preparation became a slightly viscous, opaque fluid, in which most of the mucin had become dissolved, leaving a slight deposit at the bottom of the container. It was found to give excellent results in raising the pathogenicity of the meningococcus. Later, following the directions of Miller (1934) and others, more elaborate preparations were tested, but subsequently the original method was adhered to and has given good results. On account of contaminant sporing organisms sterilization has been found necessary, and therefore autoclaving has been used; it may be noted here that

crude gastric mucin retains its virulence-increasing property after one autoclaving at 15 lb. pressure for 15 minutes, but not after two successive autoclavings.

A difficulty, also observed by Miller (1934), Miller and Castles (1936), and Proom (1937), is that marked variations occur among samples of mucin, both in regard to solubility and power to raise the pathogenicity of various organisms. We have observed that, as a general rule, the more soluble the mucin, the greater the virulence-raising properties.

Method of using Mucin.

Sub-cultures of the organism were made on blood-agar slopes or plates, and after 17 to 18 hours the growth was washed off with sterile saline, the suspension of organisms being standardized to tube 3 of the Brown's opacity standards. Without loss of time, one part of bacterial suspension was mixed with one part of the 5 per cent. mucin and 0·5 c.c. of the resulting mixture injected intraperitoneally into each of a number of mice, usually three to four. At the same time, as a control, an injection was made intraperitoneally, into a similar number of mice, of one part of the bacterial emulsion and one part of normal saline. After death cultures were made from the heart blood and peritoneal exudate.

Effect of Inoculation of Meningococci Suspended in Mucin.

Strain 22052.—This was freshly isolated from a case of meningitis. On injecting intraperitoneally into mice a dose of approximately 700 million organisms, suspended in normal saline, no lethal, nor, indeed, any apparent systemic effect was observed. However, a similar number of organisms in mucin produced a lethal result in a few hours, the mice dying of a marked meningococcal septicæmia. Further, as noted by Fothergill *et al.* (1937), the " mucin-virulence " of the strain was increased, in that by the third or fourth passage the mice died in a very few hours, and the lethal dose was reduced to one-twentieth or so of that in the initial infection.

Strain " National Collection Type I ".—Similar results were found with this strain, but it was less virulent than 22052, in that mice died only after 36–48 hours, whereas 22052 produced a fatal result in 12–24 hours.

CHEMICAL FRACTIONATION OF MUCIN.

Preparation of Pure Mucin.

The crude commercial preparations of mucin (obtained from L. Light & Co., London) were submitted to fractionation by the following method: 10 g. samples were suspended in 100 c.c. of distilled water and allowed to stand at room temperature for two hours. The solid was separated by centrifugalization and re-suspended in 100 c.c. of distilled water, and the process repeated a third time. The aqueous solution was divided into three parts. One portion was acidified with dilute acetic acid, and four volumes of alcohol were added; the precipitate was collected, washed with alcohol and ether and dried, constituting

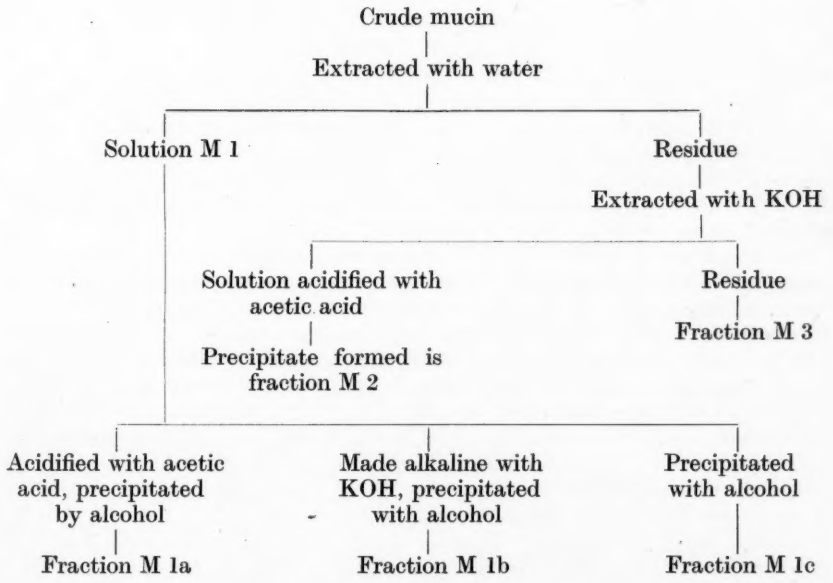
the fraction M 1a (see Table I). The second portion was made alkaline with potassium hydroxide and four volumes of alcohol were added ; the precipitate —fraction M 1b—was washed with alcohol and ether and dried. The third portion of the supernatant fluid was treated with four volumes of alcohol directly, and the precipitate collected as usual, forming fraction M 1c.

The residue remaining after aqueous extraction was treated with 50 c.c. of water and 10 c.c. of 2N potassium hydroxide, allowed to stand overnight in the refrigerator, the solid portion removed by centrifugalization and treated with water and potassium hydroxide as before, being finally processed with 5 c.c. of 2N potassium hydroxide. The combined alkaline extracts were acidified with dilute acetic acid, and the precipitate separated, washed with water until free from acid, then with alcohol and ether and dried. This fraction —M 2—is regarded as being purified mucin (see Levene, 1925).

The insoluble residue remaining after alkaline extraction was washed with water to free it from alkali, then with alcohol and ether and dried, constituting fraction M 3.

The fractionation is summarized by Table I.

TABLE I.



Preparation of Human Salivary Mucin.

Human saliva was collected and made acid with acetic acid. The precipitate so formed was washed with water, dissolved in a little sodium hydroxide solution and re-precipitated with acetic acid. The resulting product was dissolved and precipitated a second time in the same fashion. It was finally

washed with water, alcohol and ether and dried. It thus corresponds in properties and treatment to fraction M 2, obtained from commercial gastric mucin.

Hydrolysis of Mucin.

0·5 g. of mucin, fraction M 2, was suspended in 10 c.c. of N sodium hydroxide solution, and allowed to stand at 37° C. for three days, and at room temperature for a further three days. The solution, which at this stage still gave a positive biuret reaction, was then made acid by addition of trichloracetic acid, and the resulting precipitate separated by centrifugalization, washed successively with water, alcohol and ether, and dried. The product, fraction M 2*a*, gave a positive biuret reaction and a negative Molisch reaction, thus constituting the protein moiety of the mucin. To the supernatant fluid was added an equal volume of alcohol; the precipitate formed was removed, washed with alcohol, ether, and dried, to yield fraction M 2β, which gave a negative biuret and a positive Molisch reaction, being therefore regarded as the carbohydrate portion of the mucin. The remaining solution was concentrated *in vacuo*, yielding a syrupy fluid constituting the fraction M 2γ.

The amounts of the three fractions obtained were respectively 0·124 g., 0·035 g., and 2·37 g.

Trypsin Digestion of Mucin.

0·5 g. of purified mucin—fraction M 2—was suspended in 10 c.c. of water, 0·2 c.c. of liquor trypsin co. added, and then sufficient sodium hydroxide solution to dissolve the mucin and bring the pH of the solution to 8·5. The mixture was incubated at 37° C. for 20 hours, after which enough 0·25 N trichloracetic acid was added to make the solution acid to litmus. The resulting precipitate was then centrifuged out, washed with water, alcohol and ether, and dried, to give fraction M 2a (0·15 g.).

To the supernatant solution an equal volume of alcohol was added and the precipitate so formed collected, washed in the usual manner with alcohol and ether, and dried, yielding the fraction M 2b (0·045 g.). Fractions M 2a and M 2b both gave weakly positive biuret and Molisch reactions. In a repetition of the above process, in which the digestion was more complete, the residual protein fraction, M 2p—corresponding to M 2a above—was much smaller (0·054 g.), and the carbohydrate fraction, M 2c—corresponding to M 2b above—was free from biuret action, but gave a strong positive Molisch reaction. The supernatant solution remaining after precipitating the protein and carbohydrate was concentrated *in vacuo*, yielding a syrupy residue, consisting mainly of amino-acids—fraction M 2r (1·51 g.).

Method of using Mucin Fractions.

A suspension—or solution if possible—of the fraction to be tested was made in saline. To one part of this liquid was added one part of meningococcal culture suspended in saline, as described above, and 0·5 c.c. of the resulting mixture was injected intraperitoneally into each of three mice. As a control for possible toxicity, a similar amount of the fraction, made up to 0·5 c.c. with saline, was injected intraperitoneally into each of three additional mice.

In the following experiments, including the testing of some substances which might have an action similar to that of mucin, to ensure uniformity in the tests, a National Collection strain of meningococcus "Type I" was used throughout, all subcultures being made from the original, thus avoiding any errors due to passage and increase of "mucin virulence". It has been found that this particular strain, though of low virulence even with mucin, has nevertheless maintained this virulence exceptionally well.

Effect of Mucin Fractions.

The results obtained may conveniently be summarized as follows:

Crude mucin	Active in exalting pathogenicity.
Pure mucin—	
Fraction M 2	Active.
M 1	Inactive.
M 1a, M 1b, M 1c	,,
M 2*a* (protein)	Active.
M 2a (protein)	,,
M 2p (protein)	,,
M 2β (carbohydrate)	Inactive.
M 2γ	,,
M 2r	,,
Human salivary mucin—	
Fraction M 2 (pure mucin)	Active.

All the above fractions were made up in 5 per cent. solution (or suspension) in normal saline.

OTHER SUBSTANCES TESTED FOR SIMILAR PROPERTIES.

The object of this particular work was to discover if possible what types of substances possess an action similar to that of mucin. Those tested may be classified as follows, and the results of the tests are also tabulated.

Gums	Gum acacia, 10 per cent. solution	Inactive.
	Gum acacia, saturated solution	,,
Oils	Olive oil	,,
	Liquid paraffin	,,
Starches	Potato starch, 10 per cent.	,,
	Soluble starch, 10 per cent.	,,
Adsorbents	Charcoal, 5 per cent.	,,
	Kaolin, 5 per cent.	,,
	Kieselguhr, 5 per cent.	,,
Colloids	Colloidal gold	,,
	Colloidal sulphur, 5 per cent.	,,
Proteins	Edestin, 5 per cent.	,,
	Egg albumen, 5 per cent.	,,
	Gelatin, 10 per cent.	,,
	Aleuronat, 5 per cent.	,,

Carbohydrates	Pectin, 1 per cent.	Inactive.
	Pectin, 5 per cent.	,,
	Glucose, 5 per cent.	,,
	Lactose, 5 per cent.	,,
	Inulin, 5 per cent.	,,
	Dextrin, 5 per cent.	,,
	Galactose, 5 per cent.	,,
	Agar, 2 per cent. solution	Active.
	Agar, 4 per cent. solution	,,
	Agar, 10 per cent. solution	,,
	Agar, 10 per cent. cold suspension	,,
	Carbohydrate fractions from the Diphtheria bacillus, all 5 per cent.	,,
Miscellaneous	Milk	Inactive.
	Calcium chloride 3 mg. per mouse	,,
	Heat-killed suspension of *B. proteus*	,,
	B. proteus culture filtrate.	,,

Preparations of the above substances were made up as in the case of mucin fractions, and controls were used in the same fashion. Among these, the only substances capable of increasing the virulence of the meningococcus were agar and certain carbohydrate fractions isolated from the diphtheria bacillus.

The Effect of Crude Gastric Mucin on the Pathogenicity of Various Organisms.

The methods used were similar to those already described.

Staphylococcus aureus.—" Strain 4," a freshly isolated strain, proved innocuous to mice in a dose of approximately 100 million organisms injected intraperitoneally, yet when injected suspended in mucin, it uniformly produced a fatal result in 24 hours.

Precisely similar results were obtained with an old strain, " 6 ".

With a further strain, " 10 ", also old, when injected in saline two-thirds of the mice died in 36 hours, yet along with mucin, this strain produced a fatal septicæmia in all animals in 12 hours.

Streptococci.—A fairly recent strain of anaërobic hæmolytic streptococcus, non-pathogenic to mice, was first used, but mucin did not exalt its virulence.

Strain " Pope ", an aërobic hæmolytic streptococcus, however, gave striking results, small doses of organisms injected in mucin causing a fatal septicæmia in mice in 12–24 hours, yet if injected in saline, the same doses proved quite non-pathogenic. This was an old laboratory strain.

A strain from Lancefield's group " C " was tested, and gave results similar to those with " Pope ".

A strain of *Streptococcus viridans*, on repeated test, was unaffected by the mucin.

B. typhosus.—Rake (1935) stated that of 13 strains of *B. typhosus*, all had their virulence increased by mucin.

Two recently isolated strains tested by us showed no demonstrable increase of pathogenicity when injected along with mucin.

Pneumococcus.—Because of the natural susceptibility of the mouse to experimental infections with this organism, an old stock strain, originally Type 1, was used, being entirely avirulent to mice, even in very large doses. Mucin had absolutely no influence on the organism, this being in accordance with Nungester's findings (see Nungester, 1936), who stated that mucin could not lend or restore virulence to an aged, avirulent strain.

Timothy-grass bacillus, B. anthracoides, Smegma bacillus and B. coli.—No increase of pathogenicity in mice could be demonstrated with any of these organisms when injected intraperitoneally along with mucin.

DISCUSSION.

That mucin is capable of increasing the pathogenicity of certain organisms to mice is now undoubted, and the possible mode of action presents an interesting problem. The general consensus of opinion appears to be that if organisms *plus* mucin are administered intravenously or subcutaneously, little or no effect is observed (see Miller and Castles, 1936 ; Nungester *et al.*, 1932, 1936), and that both of the components of the lethal mixture require to be injected intraperitoneally. However, within limits, the two substances need not be injected simultaneously, for it is stated by the above workers that the mucin may be given up to several hours (four to five) before the injection of organisms, and a lethal effect will still result. These points have been confirmed by the writers.

Opinion in the literature ranges from crediting the mucin with the property of forming a temporary protective coating or artificial capsule for the organisms, to a specific inhibition of intracellular digestion of organisms by leucocytes. In a recent paper Keefer and Spink (1938) suggest that mucin depresses the bacteriolytic power of whole blood and of antigonococcal serum, and further, that it does not affect serum-complement, but interferes with he sensitization of the organism by antibody.

Although Benians (1924) found that *B. coli* when injected with 5 per cent. gum tragacanth intraperitoneally in rabbits was pathogenic, the present experiments show that substances such as gum acacia, etc., having similar physical properties, did not possess any action comparable with that of mucin. Again, mucin may be given in one side of the peritoneal cavity of a mouse, while organisms are given in the other side, and several hours may elapse between the injection of mucin and of organisms, by which time the mucin has possibly become too diluted to form an artificial capsule.

Nungester *et al.* (1936) stated that the relative viscosity of the mucin was the important factor in its action ; substances of widely differing viscosity have been used by us, and have proved similarly effective in raising virulence. An example may be given : our most efficient sample of mucin, " 3/5 ", was almost completely soluble in saline, and at 5 per cent. concentration appeared to be only slightly viscous ; on the other hand (*vide supra*), a 10 per cent. solution of agar, which gave a similar effect in raising virulence, was of very high viscosity. The relative viscosity of the mucin or other active compounds, therefore, seems to be of little importance in the mechanism of action of these substances.

The results with various chemical fractions of crude gastric mucin indicate that it is the protein fraction which is active in enhancing the virulence of

certain micro-organisms. The carbohydrate fraction appears to be quite inactive—a somewhat surprising finding in view of the activity of agar and of the diphtheria polysaccharides, but in keeping with the results obtained with other polysaccharides such as starch, inulin and dextrin. The polysaccharides of *C. diphtheriæ* were obtained by precipitation from aqueous solution with alcohol after removal of the protein by trypsin digestion.

Mucin itself is without toxicity to the mouse in the doses usually injected, and appears to be without significant influence on the cellular reaction when injected intraperitoneally. For example, small doses of mucin, agar and pectin were injected intraperitoneally in healthy mice, and the cellular exudate examined at intervals for 48 hours by withdrawal of samples of peritoneal fluid. No significant differences could be observed in the cellular reaction produced by active and inactive compounds.

In conclusion little can be said about the action of mucin, except that it is probably more than a mere physical effect.

SUMMARY.

(1) Gastric mucin when injected intraperitoneally into mice along with certain organisms, *i. e.* meningococcus, *Staphylococcus aureus*, and some strains of hæmolytic streptococci, which are non-pathogenic or of low virulence to this species, increases their virulence to a high degree. This influence, however, is not a general one pertaining to all types of bacteria. Thus it has not been demonstrated in our experiments with *Streptococcus viridans*, *B. typhosus*, *B. coli*, *B. anthracoides*, etc.

(2) A crude preparation of gastric mucin has been chemically fractionated, and the fractions have been tested for "virulence-increasing" activity in animal experiments with the meningococcus; only the protein fraction has been proved to be active, the carbohydrate moiety being inactive.

(3) A considerable variety of substances has been tested similarly with the meningococcus; among these agar and certain carbohydrate fractions derived from the diphtheria bacillus have proved to possess virulence-increasing properties.

(4) The action of mucin does not appear to be of a physical nature.

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SPECTROGRAPHIC OBSERVATIONS ON URINARY PORPHYRINS: THE NATURE OF A COMBINED FORM OF COPROPORPHYRIN FOUND IN URINE.

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THE frequent occurrence of a combined form of coproporphyrin in the urine of patients with acute rheumatism was reported in a previous paper (Kapp and Coburn, 1936). At that time a search of the literature disclosed the description by a number of workers of a urinary pigment with spectra and chemical properties similar to those of our coproporphyrin compound. The positions of the absorption maxima in the visible region corresponded closely with those reported for the zinc derivative of coproporphyrin. We therefore undertook a comparative study of the visible and ultra-violet spectra of our pigment obtained directly from urine, and of the zinc compound prepared *in vitro* from urinary coproporphyrin, in order to see whether these substances could be distinguished from each other.

PREPARATION OF MATERIAL.

Mixed coproporphyrins, both free and combined, were obtained from urine by ether extraction, as described previously. Samples from individual patients, usually representing the pooled extracts of a number of consecutive 24-hour specimens, were worked up separately. The quantities of pigment obtainable in this way were much too small to permit crystallization. Purification was therefore effected by successive transfers from ether to aqueous solvents until the material in question appeared spectroscopically free from contaminating pigments.

The unknown combined porphyrin was brought to a relatively pure condition by repeated washing of ether extracts with 0·5 per cent. HCl (to remove free coproporphyrin and urobilin), followed by transfer into normal ammonia, acidification with acetic acid and return to ether. This procedure effectively removed all extraneous pigments except copper coproporphyrin, which appeared as a contaminant in all preparations.

Zinc coproporphyrin was prepared by adding a few crystals of zinc acetate to an ammoniacal solution of free coproporphyrin. The reaction is quantitative and practically instantaneous, and the product goes over readily into ether after acidification of the aqueous solution with acetic acid to pH3.

Copper coproporphyrin obtained as such from urine may be freed of other urinary porphyrins by washing out an ether solution with 5 per cent. HCl. The copper compound remains in the ether (Schumm, 1927), and is further purified by shaking out into normal ammonia, followed by acidification and return to ether.

* The work reported in this communication was carried out under the W. K. Kellogg Foundation.

DETERMINATION OF SPECTRA.

The spectral range from 3750 to 4200 Å. was investigated first. In this region of the spectrum the absorption by porphyrins is so intense that very small amounts of material can be studied successfully. Dilutions were adjusted by trial to give maximum densities at a convenient level; the densities so

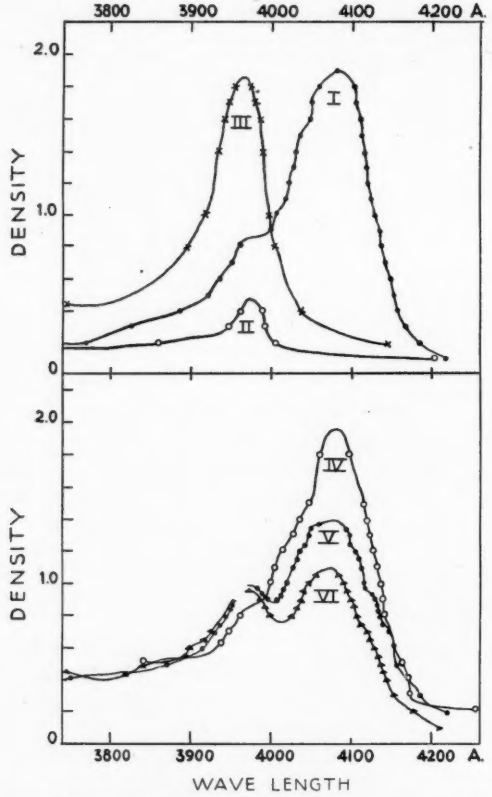

FIG. 1.—The ultra-violet absorption bands of various preparations of combined urinary coproporphyrin.

obtained are therefore without quantitative significance. The instrument used was a Hilger quartz spectrograph, model E3, equipped with a Spekker photometer and a hydrogen discharge tube source. Spectra were recorded at laboratory temperatures, which ranged between 20° and 25° C.

Several spectra of combined forms of coproporphyrin are shown in Fig. 1. Curve I is the absorption spectrum between 3750 and 4200 Å. of an ethereal solution of zinc coproporphyrin prepared from urinary coproporphyrin. The

maximum lies at 4080 Å. The secondary peak at 3970 is extraneous, since an impurity responsible for this band could be separated from the zinc preparation by chemical means. The solution was shaken with 5 per cent. HCl, and a new curve recorded for the supernatant ether adjusted to its original volume. This second curve (Fig. 1, curve II) consists of a single absorption band with its maximum at 3970 Å. As the chemical behaviour of the contaminant suggested that it might be the copper compound, a purified preparation of urinary copper coproporphyrin, in which the positions of the definitive bands in the visible at 5238 and 5613 had been checked with a spectroscope, was studied in the region of 4000 Å. Its absorption curve, shown in Fig. 1 as curve III, corresponds closely to curve II at a higher density. It seems probable that the extraneous secondary peak in curve I is due to contamination with copper, presumably present as an impurity in the reagents used.

Curves IV, V and VI represent the absorption spectra of samples of combined porphyrin obtained as such from the urines of three different individuals. These curves all have maxima at 4080 within the limit of accuracy of reading the plates. They also present secondary peaks at 3970, the relative density of which varies considerably. Preparation IV shows less evidence of copper contamination than any of the others, including I. Preparations V and VI contained so much copper that the bands in the visible region characteristic of copper coproporphyrin were easily discernible with a visual spectrometer.

The spectra of these compounds in normal ammonia were also compared, but no differences were observed. The maxima were less sharp than in ether, and lay at about 4080 Å.

Absorption data for the longer wave-lengths of the visible region were obtained with optical equipment made available through the kindness of Dr. W. F. G. Swann, Director of the Bartol Research Foundation. Spectrograms were made with a Hilger E1 spectrograph, with a quartz optical train set for the visible region and a photoflood lamp as source. Densitometric tracings of the spectrograms were made with a Krüss recording densitometer.

With the E1 spectrograph, it is possible to record the range between 3000 and 6500 Å. spread out over about 20 cm. at a single exposure. The intensity of porphyrin absorption in the vicinity of 4000 Å., however, is so high in comparison with that at longer wave-lengths that these preparations had to be run at two dilutions. Solutions in 4-cm. cells were exposed for 5 seconds to record the long-wave part of the spectrum ; 1 : 10 dilutions were then exposed for 20 seconds for the short-wave part. The resulting pairs of spectrograms were traced with the densitometer. Those from which the curves in Fig. 2 were made were all recorded on a single plate (Eastman IIIB) to insure uniform development. A mercury arc was used as reference.

In Fig. 2 are given curves of the spectra in ether of (A) a preparation of zinc coproporphyrin, and (B) a sample of combined porphyrin obtained as such from urine. The absorption curve of the urinary compound (B) shows the same maxima (4080, 5390 and 5750 Å.) as curve A, but is modified by the presence of some contaminating copper coproporphyrin. There is a distinct secondary maximum at 3970 Å., and the minima at 5200 and 5600 are less sharp than in curve A. When allowance is made for this factor, the curves from the urinary

preparation and the zinc compound are indistinguishable. All the evidence with respect to solubility, lability to strong acid, and spectral characteristics in ether and aqueous ammonia, supports the view that the previously unidentified coproporphyrin compound found in urine is the zinc derivative.

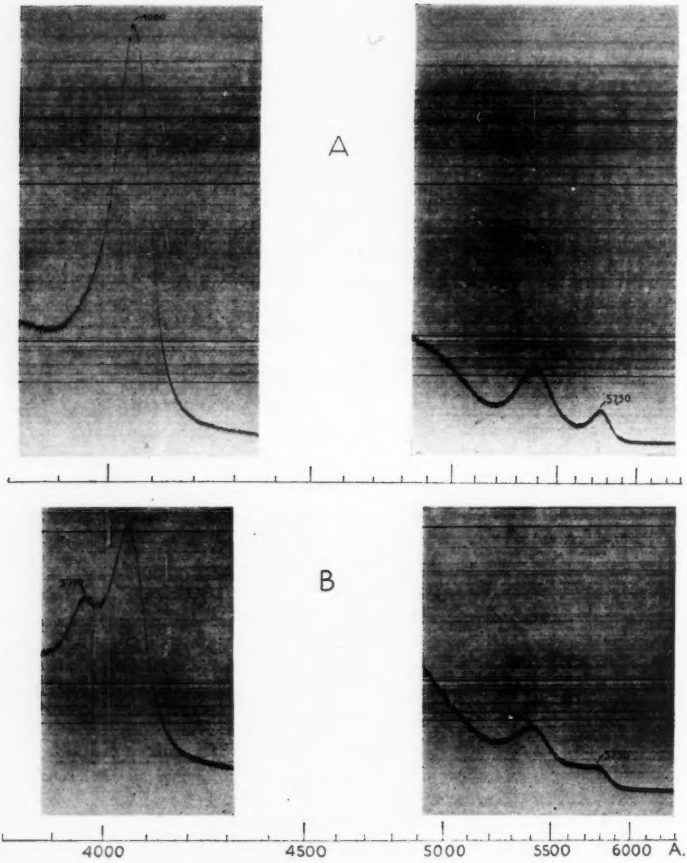

FIG. 2.—Absorption spectra in ether of (A) a preparation of zinc coproporphyrin, and (B) a sample of combined porphyrin obtained as such from urine.

The resemblance of this urinary pigment to a coproporphyrin compound which occurs in certain bacterial cultures was pointed out in an earlier paper (Kapp and Coburn, 1936). The bacterial pigment has recently been identified by Coulter and Stone (1938) as zinc coproporphyrin. They report absorption maxima at 574, 538 and 407·9 mμ.

NOTES ON THE STRUCTURE OF THE ULTRA-VIOLET ABSORPTION BANDS OF COPROPORPHYRIN AND ITS ZINC AND COPPER DERIVATIVES.

In the course of the comparative study just reported, a number of records were obtained of the ultra-violet spectrum of free coproporphyrin. These presented certain features which have not been brought out in the literature to date.

The multiple nature of the absorption bands of the porphyrins can be readily

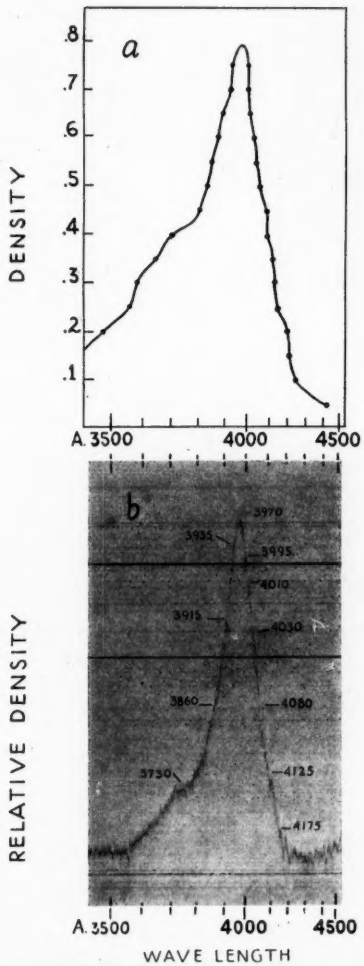

FIG. 3.—The ultra-violet absorption band of free coproporphyrin in ether, (*a*) as read from a Spekker photometer plate, and (*b*) as traced by the densitometer from a single spectrogram on the same plate.

observed in ether solutions with a visual spectrometer. In the case of coproporphyrin, the bands at 5680 and 5270 Å. show marked asymmetry, and the band at 4950 Å. is seen to consist of at least five components with easily measurable positions. Resolution of the analogous bands of etioporphyrin by cooling with liquid air was reported by Conant and Kamerling (1931). It is therefore not surprising that fine structure of considerable complexity was indicated in the ultra-violet absorption bands of several different preparations of free corpoprophyrin. A number of unresolved secondary maxima could be detected as " shoulders " or changes in slope along the sides of curves drawn from Spekker photometer plates. The reality of these maxima was shown even more definitely in densitometric tracings of the Spekker plates, which afforded a photometric check on the presence of unresolved fine structure in curves

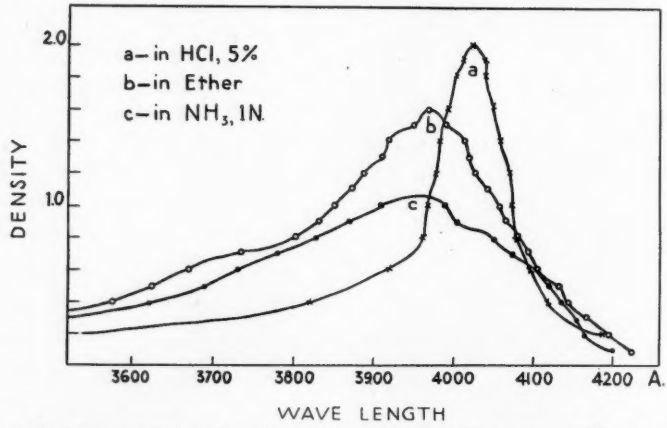

FIG. 4.—The ultra-violet absorption band of free coproporphyrin in three different solvents.

constructed from a limited series of points. In Fig. 3 are reproduced (*a*) a curve constructed from visually determined matchpoints on a Spekker photometer plate (Eastman D-C Ortho), and (*b*) the densitometric tracing of a single spectrum from the same plate. These curves are not subject to quantitative comparison with respect to density, since the density in (*a*) is plotted on a linear scale, whereas in (*b*) " density " is a complex function of plate-blackening and the order of sensitivity of the recording apparatus in the densitometer. Nevertheless, it is clear that there are definite small peaks along the sides of the tracing which correspond to changes in slope in the plotted curve.

The ultra-violet absorption curves of free coproporphyrin in three different solvents are shown in Fig. 4. Here again density levels are arbitrary, and the curves do not represent equivalent quantities of absorbing material. In HCl the absorption band is more compact than in ether or ammonia and its maximum lies at a longer wave-length. Its shape recalls the ultra-violet bands of the metallic complexes. Inasmuch as the visible spectrum of free porphyrin in acid (Stern and Wenderlein, 1934) consists of two bands quite similar in their

TABLE I.—*The Positions of Unresolved Secondary Maxima in the Ultra-violet Absorption Bands of Several Different Porphyrin Preparations. Primary Maxima are Indicated in Heavy Type.*

Solvent.	Nature of material.	Positions of primary and secondary maxima, in Ångstrøm units.																						
Ether . .	Cu coproporphyrin from urine . .	..	..	..	3905	..	3930	3955	**3972**	3985	3995	4005	4025	..	..	..	..	..	..	..	..	..	..	..
Ether . .	Zn compound prepared *in vitro* from urinary coproporphyrin*	..	..	..	..	..	..	3945	3970	..	..	..	4015	4040	4055	**4078**	4105	4115	4125	..	4145	4165	..	
Ether . .	Combined porphyrin obtained as such from urine — preparation Co*	..	..	..	..	..	..	..	3970	..	..	..	4015	4040	..	**4085**	..	4110	4125	4135	4145	4160	..	
	,, Le*	..	..	..	..	..	..	..	3972	..	..	..	4015	4032	4055	**4080**	4105	4115	..	4130	4145	..	..	
	,, Ke*	..	..	..	..	..	..	..	3970	..	..	..	..	4045	..	**4075**	4095	4115	4125	..	4145	..	..	
5 per cent. HCl	Free coproporphyrin obtained from urine	..	..	..	..	..	..	3950	..	..	3990	..	**4023**	..	4050	4070	..	..	..	..	..	..	..	
1 N.NH_3 . .	Free coproporphyrin obtained from urine	..	3760	..	..	..	..	**3950**	..	..	..	..	..	4030	..	..	4090	..	..	..	4150	..	..	
Ether . .	Free coproporphyrin obtained from urine — preparation Hi	3720	..	..	..	..	3925	..	**3975**	..	..	..	..	..	..	..	4100	..	..	..	4140	..	4180	
	,, Co	..	..	3875	..	3920	..	..	**3975**	..	..	..	..	..	..	4085	..	..	..	..	..	..	..	
	,, Ho	3730	..	3860	..	3915	3935	..	**3970**	..	3995	4010	..	4030	..	4080	..	..	4125	..	..	..	4175	
	,, Ke	3730	..	3855	..	3915	..	..	**3972**	..	..	4010	..	..	..	4080	..	4115	..	..	..	4155	4200	

* Contaminated with copper.

arrangement to those of the metallic complexes as a class (Milroy, 1918; Stern and Molvig, 1937), it is of interest that a similarity in spectral type also obtains for the ultra-violet band. (Compare curve *a*, Fig. 4, with curves I, III and IV, Fig. 1). Free porphyrin in acid and the metallic complexes in ether must have some fundamental structural feature in common which differs markedly from that of free porphyrin in non-polar solvents or in alkali.

Evidence of fine structure was also to be found in Spekker plates from the metallic derivatives. In certain plates made from synthetic and urinary zinc coproporphyrin, the presence of unresolved secondary maxima was checked with the densitometer. The approximate positions of the maxima found in all the preparations studied are summarized in Table I.

It will be seen in the table that the same secondary maxima appeared in three preparations of combined urinary porphyrin and in the curve for the known zinc compound. Another set of secondary peaks showed up in samples of free porphyrin from four different individuals. The absence of some of the peaks from some of the plates is probably due to unduly large intervals between the density levels chosen for recording with the Spekker photometer.

If we consider only the spectra made from ether solutions, it will be noted that there is a marked tendency for certain positions to recur in the spectra of chemically different preparations. For instance, the primary maximum of zinc coproporphyrin is present in the spectrum of free coproporphyrin as a secondary; the primary maxima of free porphyrin and of the copper compound are the same, within the limits of error; and several other peaks are common to both free and combined material. The presence of multiple absorption peaks in the ultra-violet bands of metallic porphyrin complexes at the temperature of liquid air was reported by Coulter* in 1934, who suggested at that time that the differences between the ultra-violet absorption spectra of complexes with different metals might be ascribed to the predominance of one or another of a common series of basic absorption bands. The present data are in accord with that suggestion. They indicate that a wealth of detail underlies the apparent simplicity of optical absorption by porphyrins in the near ultra-violet. A systematic study at low temperatures of the spectra of highly-purified samples may well yield a clue to those aspects of molecular configuration which the synthetic approach has thus far failed to elucidate.

SUMMARY.

1. Spectrographic evidence supports the view that a combined form of coproporphyrin which occurs in human urine is the zinc compound.

2. The ultra-violet absorption spectra of free coproporphyrin and of zinc coproporphyrin are recorded and discussed.

The author is indebted to Prof. H. T. Clarke for the facilities of the spectrographic laboratory in the Department of Biological Chemistry, and to Dr. Hugh H. Darby for indispensable advice and instruction in the use of optical equipment.

* Before the 2nd Summer Conference on Spectroscopy held at the Massachusetts Institute of Technology.

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THE NUTRITION OF *C. DIPHTHERIÆ* (TYPES *MITIS*, *GRAVIS* AND *INTERMEDIATE*).

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IN their studies on diphtheria, Anderson, Happold, McLeod and Thomson (1931) and Cooper, Happold, McLeod and Woodcock (1936) showed that the case death-rate was highest and the incidence of paralysis greatest with *gravis* type infections, less with *intermediate*, and least with *mitis* type infections. The inability of *gravis* strains to produce potent toxins in culture contrasted strangely with these findings. Two theories have been postulated to explain the apparent anomaly :

(1) That *gravis* strains have a more rapid invasive power than other strains (McLeod and Orr, unpublished observations).

(2) That the *gravis* type is more toxigenic *in vivo* than *in vitro* compared with the *intermediate* and *mitis* types.

This second hypothesis is based on the knowledge that the production of an antigen by an organism normal to one set of cultural conditions may be inhibited by controlled variations in those cultural conditions. A comparative study of the nutrition of the three main types of *C. diphtheriæ*, and of the factors which influence their toxin production in synthetic or semi-synthetic media, might throw light on this problem. It is important to establish, not only whether their growth requirements differ, but whether they require different nutritional conditions to produce their most potent toxins. Progress in this direction has been made through the work of Mueller and his collaborators in America (Mueller *et al.*, 1933 and onwards), together with Pappenheimer's studies on toxin production (1937). Mueller's aim has been to define chemically the requirements of the diphtheria bacillus, which would permit growth equal or superior to that obtained with the normal peptone-infusion

broth. They have confined themselves to the study of a very limited number of organisms (four)—two strains of the " Park-Williams No. 8 ", the " Allen " and the " Yü " strains of *C. diphtheriæ*, which, as far as we have been able to ascertain, are all *mitis* or atypical *mitis* types. The nutrition of the *gravis* and *intermediate* types has not as yet been investigated.

Mueller and his co-workers in their nutritional studies have used two basal media :

(1) An acid hydrolysate of commercial casein, to which cystine, glutamic acid, sodium lactate and inorganic salts and occasionally tryptophane were added.

(2) A medium replacing the casein hydrolysate portion of (1) by eight amino-acids : *l*-tryptophane, *l*-cystine, *l*-histidine hydrochloride, *dl*-phenylalanine, *dl*-methionine, glycine, *dl*-valine, *d*-glutamic acid, and sometimes replacing the lactate as an energy source by sodium acetate, glycerol, or in part by ethyl alcohol.

To the above basal media a whole tissue-extract had to be added in small amount before reasonable reproducible growth could be obtained.

They showed that the active principles present in tissue extractives and similar concentrates were also present in a 95 per cent. alcohol-soluble fraction of liver. Finally, work on the extractive portion has led to the recognition of at least three substances required as growth factors by the strains tested. These are nicotinic acid, β-alanine and pimelic acid. These growth factors in appropriate amounts were adequate substitutes for the meat extracts in their ability to promote the growth of the organisms tested. Nicotinic acid was actually isolated from the active liver fraction, whilst the pimelic acid was obtained from an active urine concentrate. Concerning the third factor, β-alanine has not actually been isolated from the liver extract, but Mueller had evidence that it was of the same chemical nature as β-alanine. It had also been previously shown to increase the growth of certain yeasts by Williams and Rohrman (1936).

With the incorporation of these three factors in the casein hydrolysate medium, Mueller has obtained growths approaching to two-thirds of the maximal growth of the diphtheria bacilli tested when using a whole tissue extract added to the same basal medium.

There still remains the possibility, however, of the commercial casein hydrolysate contributing additional unknown factors to the basal medium of Mueller which, whilst unable themselves to permit appreciable growth, might nevertheless play their part when the three essential growth factors are added. A medium composed entirely of synthetic chemicals, and capable of supporting the growth of the organism on continued subculture, is the arbitrary standard by which one can judge that all the essentials for the growth of that organism have been discovered.

In the present study we have grown numerous strains of *C. diphtheriæ* (*mitis*, *gravis* and *intermediate* types) in media which, whilst they still contain some amino-acids of natural origin, are chemically more defined than those so far reported. Since differences in the nutrition of these types became apparent even in these simple media, it seems worth while to publish the results so far obtained.

EXPERIMENTAL.

Technical Details.

Apparatus.

All glassware has been cleaned in chromic acid, washed several times with glass-distilled water, and finally boiled in the latter. The cotton-wool plugs of the culture-tubes were wrapped with cotton gauze to prevent strands of wool falling into the medium—a precaution previously adopted by Fildes and Knight. The culture tubes were of " Monax " glass, dimensions 6 in. × $\frac{3}{4}$ in.; each tube contained 5 ml. of medium. The tubes were placed in trays which gave a standard inclination, each tube being raised $\frac{1}{2}$ in. at the plug end.

Strains.

The strains of *C. diphtheriæ* used were isolated during the course of the investigation from cases of clinical diphtheria in Manchester, Liverpool and Leeds, except for the " Allen " strain, which we obtained through the courtesy of Prof. J. H. Mueller. Each strain was passed by platinum loop inoculation through five serial cultures in media under test before taking any records of growth. Most of the strains have been tested for virulence (in the guinea-pig), and are positive in this respect. All strains have been tested for fermentative reactions and for characteristic growth in bouillon and on solid media; these observations have enabled us to classify them as *mitis*, *gravis* or *intermediate* types.

Comparison of Bacterial Growth.

From the nature of the growth of *C. diphtheriæ*, turbidimetric methods are useless for comparisons of growth except with a limited number of strains. For this reason we have used the Pregl micro-Kjeldahl as adapted for the estimation of bacterial nitrogen by Mueller (1935), to compare the amounts of bacterial growth in any one particular medium. Growth was determined after five days' incubation at 37° C. unless otherwise stated.

Purity and Sources of Chemicals Used for Media.

The inorganic salts used were all of " A.R." quality. The sodium lactate initially used was the B.D.H. " syrupy " material, but this was soon replaced by A.R. lactic acid, neutralized with A.R. sodium hydroxide. The source of the amino-acids used during the course of the investigation is indicated in the formulæ of the media which follow. Pimelic acid was prepared by the reduction of salicylic acid, according to the method of Walker and Lumsden (1901). Nicotinic acid and β-alanine of synthetic origin were used.*

Glass-distilled water has been used in the preparation of all media. After adjusting the pH to 7·6, the media were sterilized by filtration through a glass Seitz filter (Knight and Fildes, 1936).

* Obtained from Light & Co., 6, Featherstone Buildings, London, W.C. 1.

Description of Media and Growth Results Obtained.

Medium I.

The composition was as follows :

l-tryptophane	1 mg.
l-cystine	1 ,,
l-histidine HCl	1 ,,
s *dl*-phenylalanine	1 ,,
s *dl*-methionine	1 ,,
s glycine	5 ,,
s *dl*-valine	10 ,,
s *dl* Na glutamate	50 ,,
Na lactate A.R.	150 ,,
NaCl	50 ,,
$Na_2HPO_4.12H_2O$	25 ,,
KH_2PO_4	3·5 ,,
$MgSO_4.7H_2O$	3·0 ,,
Distilled water to	10 ml.
pH adjusted to	7·6.

" s " denotes amino-acids of synthetic origin ; the remainder were Hoffmann-La Roche products.

In Medium I the *mitis* and *gravis* types grew slightly in the first subculture, but failed to grow in the second subculture ; none of the *intermediate* strains grew in this medium.

Medium II.

As a result of Mueller's findings regarding the three growth factors—nicotinic acid, β-alanine and pimelic acid—these were added to Medium I, giving Medium II. The growth-factors were added in the following amounts :

Pimelic acid	1 γ	per 10 ml. of medium.
Nicotinic acid	10 γ	
β-alanine	10 γ	

Medium II maintains the growth of most *mitis* and *gravis* strains, results being given in Table I, with growth in bouillon for comparison.

In earlier experiments in which glutamic acid of natural origin and the so-called syrupy sodium lactate were used, *mitis* and *gravis* strains grew in Medium I (in some cases better than in Medium II). This indicated the presence of growth-promoting substances in these natural materials, the lactate containing more than the glutamic acid—probably since it is obtained by fermentation processes. In view of this experience the materials used in subsequent media have had their purity very closely investigated.

Growth of *intermediate* strains in Medium II was not supported on continued subculture. This failure of the *intermediate* strains to grow on Medium II, which was sufficient to support the growth of most *mitis* and *gravis* strains, gave rise to a problem which has to some extent been elucidated by a combination of two lines of approach.

TABLE I.—*Growth of* mitis *and* gravis *Strains in Medium II.*

Strain.		Bacterial N in mg. per 10 ml. medium.	
		Bouillon.	Medium II.
mitis	691	..	0·44
	Gallagher	0·85	0·67
	Allen	2·00	0·44
	MM6	0·46	0·03
	MM5	..	0·34
	82	0·51	0·78
	MM4	1·12	0·47
Atypical *mitis*	75391	0·93	0·50
	P.W. δ 3	0·64	0·34
	P.W. δ 1058	0·63	0·42
gravis	688	0·94	0·51
	Freeman	1·51	0·36
	MG7	0·56	0·35
	MG11	0·64	0·20
	710	0·88	0·13

Casein Hydrolysate and "Total Amino-acid" Media.

It was found that a commercial casein hydrolysate medium, as given below, was capable of supporting the growth of the *intermediate* strains.

*Casein HCl hydrolysate	10 g.
l-cystine	0·1 g.
Na(mono) glutamate	5·0 g.
l-tryptophane	0·1 g.
s *dl*-methionine	0·1 g.
s *dl*-phenylalanine	0·1 g.
Na lactate (A.R.)	15 g.
Mineral salts as in Medium I.	
Distilled water to	1 litre.

However, a "total amino-acid" medium (Table II) containing most of the amino-acids likely to be present in the casein hydrolysate, together with lactate, Mueller's essentials and salts failed to support the growth on continued subculture. Further, it was found that if the commercial casein was carefully purified by Hammersten's method (Hammersten and Hedin, 1915), after previous exhaustive extraction with organic solvents, hydrolysed with A.R. HCl, and clarified with norite charcoal (which had also been subjected to a rigorous purification process), and this material was used for making the "casein hydrolysate" medium, the medium was unable to support growth effectively, even with the addition of Mueller's essentials.

* The casein hydrolysate was prepared from commercial casein by refluxing for 48 hours with concentrated HCl, followed by removal of the acid under reduced pressure. The residue thus obtained was made up as a 20 per cent. solution (calculated on the original weight of casein taken), and then clarified with Norite charcoal.

TABLE II.—*Total Amino-acid Medium.*

l-tryptophane	0·1 g.	s *dl*-aspartic acid	0·1 g.
l-cystine	0·1 g.	s *dl*-serine	0·1 g.
s *dl*-phenylalanine	0·1 g.	s *dl*-tyrosine	0·1 g.
s glycine	0·5 g.	s *dl*-lysine di HCl	0·1 g.
s *dl*-methionine	0·1 g.	*l*-histidine HCl	0·1 g.
s *dl*-valine	0·5 g.	*d*-arginine	0·1 g.
s *dl*-glutamic acid	5·0 g.	*dl*-proline	0·1 g.
s *dl*-alanine	0·1 g.	*l*-oxyproline	0·1 g.
s *dl*-leucine	0·5 g.	**l*-threonine	0·1 g.
s *dl-iso*leucine	0·1 g.	Na lactate	15·0 g.
s *dl-nor*leucine	0·1 g.	Salts as in Medium I.	

"s" denotes amino-acids of synthetic origin.

From this it is clear that some substances essential for the growth of *intermediate* strains are present in commercial casein and are removed by the purification processes.

Mineral Matter of Casein and Inorganic Salts.

One of the chief results of the casein purification is an almost complete removal of mineral matter, which in the commercial casein used amounted to approximately 9 per cent. by weight (as determined by ignition). Wright and Papish (1929) obtained a spectrographic analysis of the mineral content of casein with the approximate relative amounts of each element. A more detailed analysis of milk products has recently been carried out by Dingle and Sheldon (1938). Accordingly, a solution containing the elements as found by the above workers and in relative amounts based on the observation that commercial casein contains about 9 per cent. mineral matter as ignited ash was made up. This salt mixture conforms closely to the formula used by Wesson (quoted by Seifriz, 1936) for a mineral mixture used in synthetic diets for experimental animals. The mixture is given below:

Salt Mixture C.

KCl (A.R.)	1·20 g.	KI (A.R.)	0·001 g.
KH_2PO_4 (A.R.)	3·10 g.	HBO_3 (A.R.)	0·001 g.
$Ca_3(PO_4)_2$ ("B.D.H. precipitated")	1·49 g.	$Sr(NO_3)_2$	0·001 g.
		Li_2SO_4	0·001 g.
$CaCO_3$ (A.R.)	2·10 g.	Na_2SiO_3	0·001 g.
$MgSO_4$ (A.R.)	0·90 g.	$TiSO_4$	0·001 g.
$FePO_4$	0·147 g.	Na_3VO_4	0·001 g.
$MnSO_4$ (A.R.)	0·002 g.	$RbSO_4Al_2(SO_4)_3\,24H_2O$	0·001 g.
$K_2Al_2(SO_4)_4\,24H_2O$ (A.R.)	0·0009 g.	Distilled water to	1 litre.
$CuSO_4\,5H_2O$ (A.R.)	0·0039 g.		

Such a solution contains fifty times as much mineral matter as the 0·2 per cent. commercial casein hydrolysate medium; thus 0·2 ml. of the above solution C was used to each 10 ml. of medium.

* We are grateful to Prof. W. C. Rose, of Illinois, for a sample of this amino-acid. The other amino-acids were Hofmann-La Roche products.

On adding the above mineral solution C to the total amino-acid medium, the growth of the *intermediate* strains was still not supported. However, when the three growth factors—pimelic acid, nicotinic acid and β-alanine—were added to the total amino-acid medium along with the mineral solution C, the resulting medium was capable of supporting the growth of all the *intermediate* strains. The growth obtained with this medium is quite equal to that obtained with bouillon, as will be seen from Table III.

TABLE III.—*Growth of* intermediate *Strains on the "Total Amino-acid" Medium + Mueller's Essentials + Salt Mixture C.*

Strain.	Bacterial N. in mg. per 10 ml. medium.	
	Bouillon.	T.A.A. + M + C.
Hat.	0·38	0·39
Shore	0·52	0·53
372	..	0·45
Tonsil	..	0·50
38	0·27	0·45
Gold	0·43	0·30
Thompson	0·30	0·48

It should be clearly understood that in the above medium the addition of salt mixture C to the "total amino-acid" medium and the three growth factors makes all the difference between growth and no growth. It is quite probable that some of the minerals present in salt mixture C are not essential for the growth of the *intermediate* strains, but which of these salts are necessary has not yet been determined.

TABLE IV.—*Growth of Some* mitis *and* gravis *Strains.*

Strain.		Bacterial N; mg./10 ml. medium.		
		Bouillon.	II.	II + C.
mitis	691	..	0·44	..
	Gallagher	0·85	0·67	1·23
	Allen	2·00	0·44	1·67
	MM6	0·46	0·03	0·65
	MM5	..	0·34	1·06
	82	0·51	0·78	0·94
	MM4	1·12	0·47	..
Atypical *mitis*	75391	0·93	0·50	2·3
	P.W. δ 3	0·64	0·34	1·4
	P.W. δ 1058	0·63	0·42	1·8
gravis	688	0·94	0·51	1·28
	Freeman	1·51	0·36	1·88
	MG7	0·56	0·35	1·33
	MG11	0·64	0·20	1·64
	710	0·88	0·13	1·96

Although the *mitis* and *gravis* types had been grown on Medium II without any complex salt mixture, it was decided to determine the effect, if any, of salt mixture C on these types when used in conjunction with Medium II. The

results are given in Table IV, with some growth records obtained in bouillon and Medium II. It will be seen that mineral mixture C increases the amount of growth (in some cases markedly so), even compared with bouillon.

CONCLUSIONS AND SUMMARY.

The three main types of *C. diphtheriæ* have been grown on media more completely synthetic and chemically defined than previously recorded ; differences in nutritional requirements exist between the *intermediate* strains on the one hand and the *gravis* and *mitis* strains on the other.

The great majority of the strains of the *gravis* and *mitis* types can be grown on a medium containing eight amino-acids, together with lactate, the three growth factors (nicotinic acid, β-alanine and pimelic acid) and mineral salts as used by Mueller. Our findings in this respect confirm and extend the work of Mueller.

The *intermediate* strains do not grow under the above conditions ; they may, however, be successfully grown in a medium containing (*a*) all the available amino-acids, (*b*) Mueller's three essentials, and (*c*) a mixture of certain minerals, together with lactate. The growth obtained in such a medium may even exceed that obtained with an unpurified casein hydrolysate medium, and compares favourably with that obtained in bouillon.

The mineral mixture so necessary for the growth of the *intermediate* strains increases the total growth of the *mitis* and *gravis* strains when added to Mueller's medium.

We are glad to record our indebtedness to those who have assisted us in our studies: To Profs. J. W. McLeod, H. B. Maitland, H. D. Wright and J. H. Mueller ; to Drs. J. Gordon and K. E. Cooper, and to the Wellcome Laboratories for strains of *C. diphtheriæ*. To L. Davies for assistance with micro-nitrogen determinations, and to H. Lawrence for the care he has taken in the cleaning of glassware, and to the Medical Research Council, who have assisted us with a grant for materials.

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FURTHER OBSERVATIONS ON FILTERABLE TUMOURS INDUCED IN FOWLS BY INJECTION OF TAR.

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IN a previous communication four experiments were described, comprising 21 Plymouth Rock fowls, in 13 of which tumours were found after the injection of tar. Four tumours were propagated in series, giving five histological types, and of these three were transmitted by means of cell-free filtrates (McIntosh, 1933), details of which are tabulated below. In this communication the completed histories of the five original propagated tumours, (Nos. 1–5) are given, together with further experiments in which new tar-induced tumours were obtained (Tumours Nos. 6–10). Further evidence will be given on the nature of these tumours, particularly with regard to filterability.

FIRST SERIES OF TUMOURS.

Further Propagation of Tumours, Nos. 1–5.

The completed histories of the propagation of the earlier tumours are summarized in Table I. All the routine passages of tumours were done with dilute emulsions, which were prepared by grinding the tumour with sand and diluting with saline to a final concentration of 2·5 g. tumour in 100 c.c. In some of the earlier passages, however, thicker emulsions were used, containing up to 50 per cent. tumour. Emulsions were also frequently treated at room temperature for 2½ hours with trypsin (5·0 per cent. liquor trypsin co., Allen & Hanbury). This procedure resulted in more vigorous tumour growth and made the tumours more readily filterable. All Berkefeld filters used in our experiments are tested bacteriologically immediately after use by passing through the filter 1·0 c.c. of an 18-hour broth culture of *B. prodigiosus* in 20 c.c. of broth and incubating the entire filtrate. The tumours maintained their distinctive histological characters throughout, although Tumour 5 became rather more myxomatous. They also maintained their distinctive rates of growth, which may be judged by the average interval at which transmission was necessary. The non-filterable tumours, Nos. 1 and 4, grew very slowly, whereas the filterable tumours, although growing more rapidly, varied widely in their growth-rates. Fig. 1 shows the first 17 passages of Tumour 3, and also a subsidiary passage line in which for three passages the tumour was replaced by leukæmic changes. This finding will be referred to later.

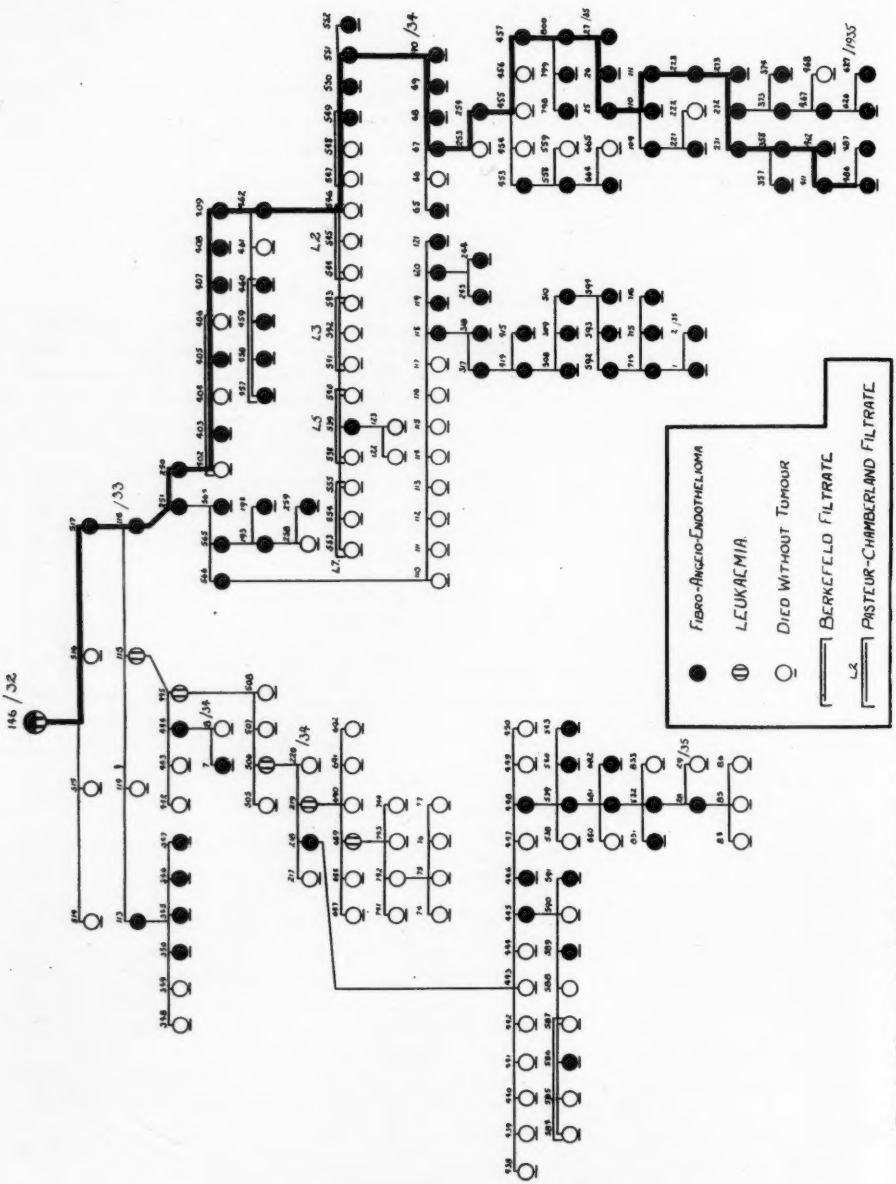

FIG. 1.—Tumour 3: First 17 passages showing substrain in which the tumour was replaced by leukæmia for 3 passages.

TABLE I.—*Propagation of Tumours 1–5.*

Tumour.	Primary.	Number of passages.	Average interval between passages (days).	Passages at which Berkefeld filtrates— succeeded.	failed.
1	No. 388, Expt. 1	3	82	..	..
2	No. 389, Expt. 1	42	33	4th, 9th	7th
3	No. 146, Expt. 2	31	51	3rd, 4th, 5th, 6th, 7th	..
4	No. 520, Expt. 3	8	85	..	4th
5	No. 520, Expt. 3	81	25	6th, 23rd, 81st	..

SECOND SERIES OF TUMOURS.

Production of Primary Tumours 6–10.

The induction experiments are summarized in Fig. 2, which includes the earlier findings in Experiment 4. In the later experiments tumours were found in association with tar injections in 6 of the 19 fowls which survived 10 weeks.

Experiment 4 (85/33).

Six Plymouth Rock fowls were given an injection of 0·25 c.c. of Neepsend tar in the right breast, and 14 days later a similar injection in the left breast. Tumours were found in 5 fowls, three of which were described in the first article and two, found in the remaining fowls, are described below.

Fowl No. 180/33.—A tumour was palpable in the right breast 142 days after the first inoculation. When the fowl was killed at 183 days the tumour measured 1 in. by ¾ in. It was encapsulated in the breast-muscle and firm in consistency. There was a small deposit of tar in the centre of the tumour. There was a similar but smaller tumour in the left breast. Nothing else abnormal was found. *Microscopical:* The tumour from the right breast is a cellular fibrosarcoma consisting of loosely-packed spindle-cells arranged in large interlacing bundles with a moderate degree of differentiation. The tumour is infiltrated by a number of eosinophils. The tumour from the left breast is of the same type, but more fibrous.

Fowl No. 183/33.—A tumour could be palpated in the right breast 154 days after the first inoculation. When the fowl was killed at 202 days the tumour measured 2½ in. by 2 in. (Fig. 4). It was firm and fibrous, and there were deposits of tar at the periphery and in the centre of the tumour. In the left breast there were small white nodules surrounding tar deposits. Nothing else abnormal was found. *Microscopical:* The tumour is a rather cellular fibrosarcoma consisting of large bundles of closely-packed spindle-cells, which, although showing some irregularity in shape, are mostly elongated (Fig. 5). There are numerous mitoses. The periphery of the tumour shows tar with giant-cells, accumulations of lymphocytes and a marked fibroblastic reaction (Figs. 6 and 7). Similar tar granulomata are to be seen in the left breast, with proliferation of the sarcolemma sheaths.

The tumour from the right breast of Fowl No. 180/33 and the tumour from Fowl No. 183/33 are Nos. 6 and 7 of our series of tar-induced tumours.

FIG. 1.—Tumour 3: First 17 passages showing substrain in which the tumour was replaced by leukæmia for 3 passages.

Experiment 5 (143/33).

Six Plymouth Rock fowls were given 4 injections of 0·1 c.c. of 5·0 per cent. Passey tar in lard in the right breast at weekly intervals. Tumours were found in two fowls.

Fowl No. 332/33.—A tumour could be palpated in the right breast 72 days after the first inoculation. When the fowl died at 271 days the tumour measured $2\frac{1}{2}$ in. by $1\frac{1}{2}$ in (Fig. 8). It was whorled, fibrous and hard, and tar

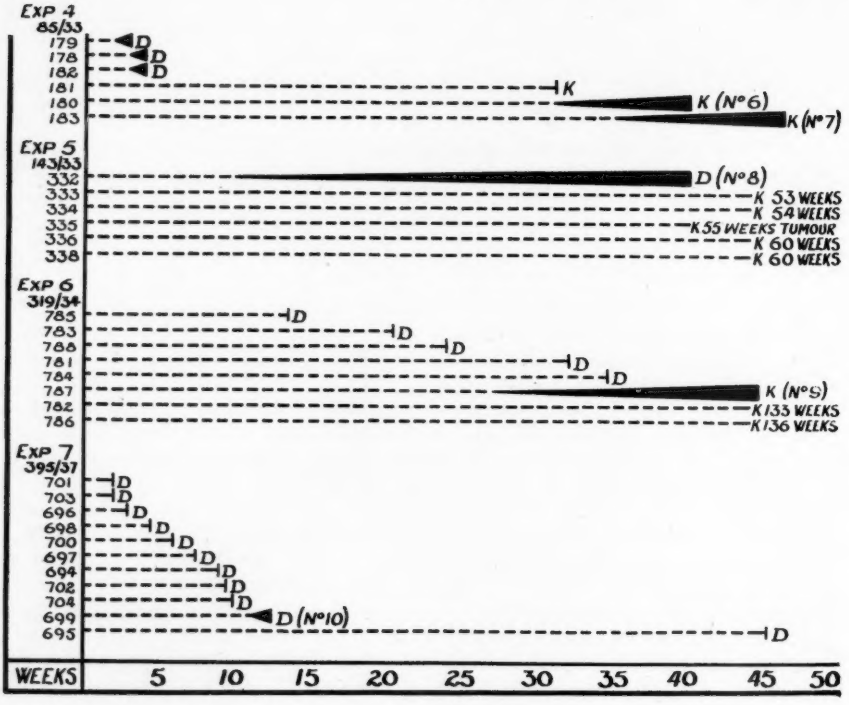

FIG. 2.—Induction of primary tumours. Tumours are represented graphically.

could be seen at the periphery and in the substance of the tumour. The liver was greatly enlarged, measuring 6 in. in its longest axis, and was in parts entirely replaced by large, very hard tumour nodules. Smaller tumour nodules were found throughout the rest of the liver (Fig. 9). *Microscopical:* The tumour is a vascular fibrosarcoma consisting of interlacing bundles of long spindle-cells with elongated nuclei (Figs. 10 and 11). There is a fine network of collagenous fibres and small areas of myxomatous degeneration. The tumours in the liver are similar to the breast tumour, but are less differentiated and show pleomorphism of the tumour-cells, some being very large and some

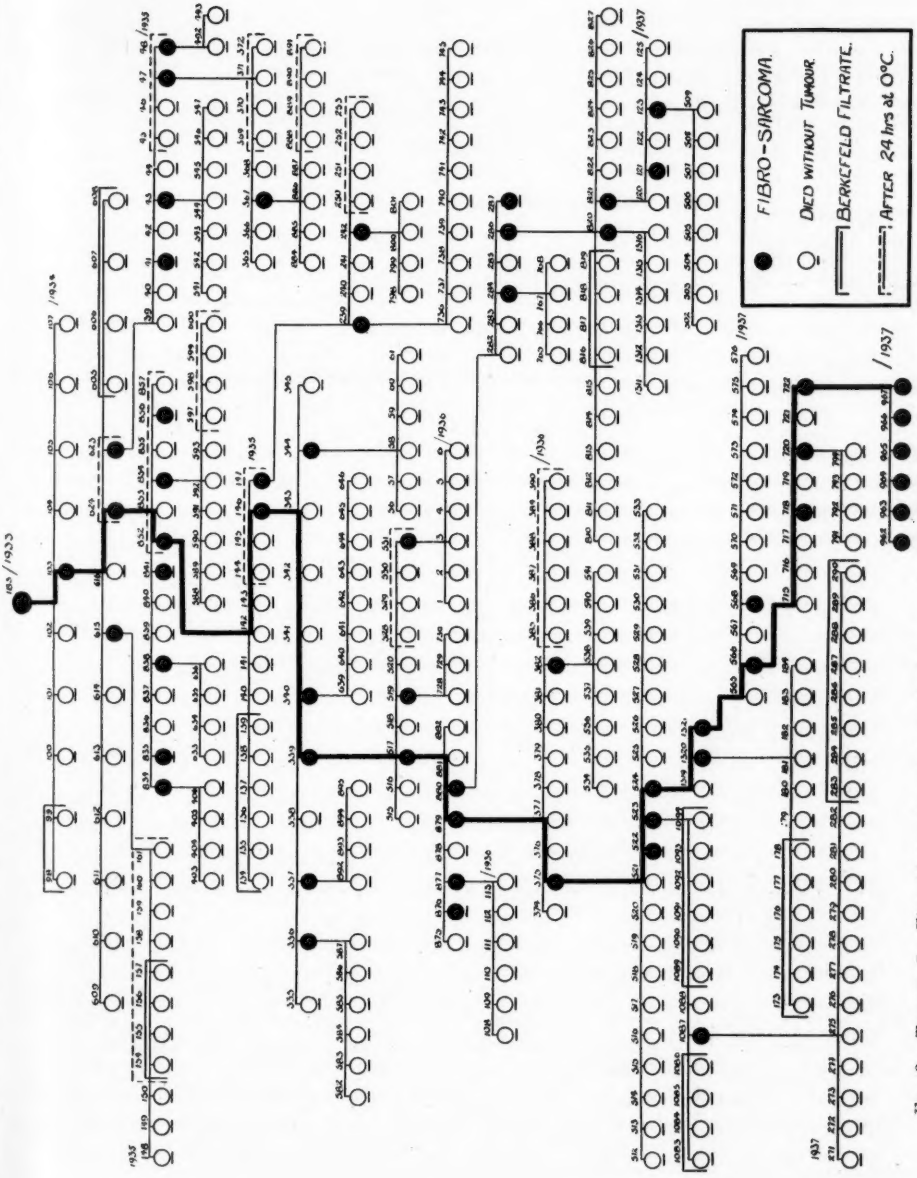

FIG. 3.—Tumour 7: First 13 passages to show the large numbers of fowls which were required to maintain this tumour.

forming giant-cells. The breast tumour is infiltrated by a few eosinophils. There is no evidence of leukæmia.

Fowl No. 335/33.—The fowl died at 385 days with no visible evidence of tumour-formation. *Microscopical:* A very small tumour (2 mm.) was found in the muscle of the right breast. It is a fibrosarcoma consisting of elongated spindle-cells of irregular size and shape, with hyperchromatic nuclei.

The tumour from Fowl No. 332/33 is No. 8 of our series.

Experiment 6 (319/34).

Eight Plymouth Rock fowls were given 4 injections of 0·1 c.c. of 25 per cent. Passey tar in lard in alternate breasts at weekly intervals. Tumours were found in one fowl.

Fowl No. 787/34.—A tumour could be palpated in the right breast 167 days after the first inoculation. When the fowl was killed at 315 days two tumours were found in the muscle of the right breast (Figs. 18 and 19). They were firm and fibrous, and measured 2 in. by 1 in. and 1 in. by 1 in. There were deposits of tar in the centres of both. A small tumour was found in the muscle of the left breast, and there was a small tumour nodule in the liver (Fig. 22). Nothing else abnormal was found. *Microscopical:* The larger tumour from the right breast is a cellular fibrosarcoma consisting of well-differentiated bundles of spindle-cells with numerous mitoses (Figs. 20 and 21). In the substance of the tumour there are small deposits of tar, with foreign-body giant-cells surrounded by vascular fibroblastic tissue. There are accumulations of lymphocytes at the periphery of the tumour. The smaller tumour from the right side is similar in type, but has more tar in its substance. The tumour from the left breast is also a fibrosarcoma, but is more cellular and pleomorphic. The nodule in the liver is similar in appearance to the primary tumour (Fig. 23).

The larger tumour from the right breast of Fowl No. 787/34 is No. 9 of our series.

Experiment 7 (395/37).

Eleven Plymouth Rock fowls were given 4 injections of 0·1 c.c. of 5·0 per cent. Passey tar in lard in alternate breasts at weekly intervals. Tumours were found in one fowl.

Fowl No. 699/37.—When the fowl died 83 days after the first inoculation tumours were found in both breasts. The right tumour measured $\frac{3}{4}$ in. by $\frac{1}{2}$ in. and the left tumour 1 in. by $\frac{1}{2}$ in. They were opaque and firm, the right tumour being rather firmer than the left. Tar could be seen in both tumours. *Microscopical:* The tumour from the left breast is a cellular fibrosarcoma consisting of well-differentiated bundles of long spindle-cells with numerous mitoses. The tumour from the right breast is an undifferentiated spindle-cell sarcoma, in which the cells are divided into groups by coarse collagenous bands resulting from proliferation of the muscle-sheaths. There is a diffuse eosinophil infiltration of both tumours.

The tumour from the left breast of Fowl No. 699/37 is No. 10 of our series.

TABLE II.—*Propagation of Tumours 7–10.*

Tumour.	Primary.	Number of passages.	Average interval between passages (days).	Passages at which Berkefeld filtrates—	
				succeeded.	failed.
7	No. 183, Expt. 4	21	(1st to 10th) 121 (11th to 21st) 41	..	1st, 2nd, 3rd, 4th, 9th, 10th, 11th, 14th
8	No. 332, Expt. 5	8	(3rd to 8th) 62	4th, 6th	5th, 7th
9	No. 787, Expt. 6	42	25	4th 5th, 41st	3rd
10	No. 699, Expt. 7	6	30	..	5th

Propagation in Series of Tumours 6–10.

The serial propagation of Tumours 7 to 10 are summarized in Table II, and the following description will be confined to the earlier passages and to any special features.

Tumour 6 (from Fowl No. 180/33, Experiment 4).

Seven fowls were inoculated with ordinary and trypsinized emulsions of the primary tumour. No tumours resulted, although 6 fowls lived for over 7 months. Two fowls inoculated with a Berkefeld filtrate were also negative.

Tumour 7 (from Fowl No. 183/33, Experiment 4).

Eight fowls were each inoculated with 1·5 c.c. of an emulsion of the primary tumour in the right breast and 1·5 c.c. of a trypsinized emulsion in the left breast. In one fowl a tumour could be palpated in the left breast at 85 days. At 210 days, when the fowl was killed, the tumour measured $2\frac{1}{2}$ in. by $1\frac{1}{2}$ in. In the right breast there was a smaller tumour measuring $\frac{1}{2}$ in. by $\frac{1}{2}$ in. The other fowls were negative. The first thirteen passages of this tumour are shown in Fig. 3, which shows the difficulties which are encountered in maintaining these tumours. The first ten passages were made with tumour emulsion. At the 2nd, 3rd, 4th, 5th and 6th passages emulsions were made from tumour material that had been kept in the refrigerator for 24 hours. They were more successful than fresh emulsions on three occasions, and in one passage succeeded where the fresh emulsion failed. At the 11th passage four attempts with emulsions gave no result, and since then the tumour has been propagated with inoculations of minced tissue. In the later passages twelve attempts have been made with tumour emulsions in 63 fowls and only once has a tumour emulsion produced a tumour.

Filterability.—Berkefeld filtrates have consistently failed to give tumours. A sand and paper-pulp filtrate tested at the 11th passage, and an emulsion of frozen tumour, tested at the 18th passage, both failed.

Histology.—Tumour 7 has retained the characters of the primary tumour with slight variations in the amount of myxomatous degeneration and collagenous content. Older tumours, however, are firmer and show coarser collagenous fibres, sometimes with areas of hyaline degeneration.

EXPLANATION OF PLATES.

FIG. 4.—Tumour 7 : Primary tumour in breast-muscle showing tar deposits at the periphery. (Fowl No. 183/33, Experiment 4.)

FIG. 5.—Tumour 7 : Section of primary tumour showing a cellular fibrosarcoma consisting of closely-packed spindle-cells. × 100.

FIG. 6.—Tumour 7 : Section from periphery of primary tumour showing tar deposits. × 100.

FIG. 7.—The same, showing foreign body giant-cells, mononuclear cells and fibroblastic reaction. × 450.

FIG. 8.—Tumour 8 : Primary tumour in breast-muscle showing tar deposits at the periphery. (Fowl No. 332/33, Experiment 5.)

FIG. 9.—Tumour 8 : Multiple liver metastases from primary tumour.

FIG. 10.—Tumour 8 : Section of primary tumour showing bundles of long spindle-cells. × 100.

FIG. 11.—The same, showing long spindle-cells with elongated nuclei. × 450.

FIG. 12.—Tumour 8 : Section of leukæmic tumour in first passage, showing infiltration of muscle by closely packed myeloid cells. × 100.

FIG. 13.—The same, showing characters of myeloid cells, many of which show eosinophil granules. × 450.

FIG. 14.—Tumour 8 : Section of leukæmic tumour from second passage showing fibrosarcomatous stroma. × 100.

FIG. 15.—The same, showing primitive myeloid cells in fibro-sarcomatous stroma. × 450.

FIG. 16.—Tumour 8 : Section of tumour from Berkefeld filtrate in the sixth passage. The tumour is more open in structure than the primary tumour (Fig. 10). × 100.

FIG. 17.—The same, showing similarity of cells to those of the primary tumour (Fig. 11). × 450.

FIG. 18.—Tumour 9 : Primary tumour in breast-muscle showing tar in centre. (Fowl No. 787/34, Experiment 6.)

FIG. 19.—Tumour 9 : Smaller tumour found lower down in the same breast as the primary tumour, also showing tar in centre.

FIG. 20.—Tumour 9 : Section of primary tumour showing a cellular fibro-sarcoma. × 100.

FIG. 21.—The same, showing characters of tumour-cells. One mitosis is shown. × 450.

FIG. 22.—Tumour 9 : Liver metastasis from primary tumour.

FIG. 23.—Tumour 9 : Section of liver metastasis showing liver tissue at upper part of figure. × 100.

FIG. 24.—Tumour 9 : Section of tumour from 7th passage. Although it is more open in structure, it is similar in appearance to the primary tumour (Fig. 20). × 100.

FIG. 25.—The same, showing similarity of tumour-cells to those of primary tumour (Fig. 21). × 450.

FIG. 26.—Tumour 3 : Section of liver from first leukæmic passage, showing infiltration of liver with formation of leukæmic nodules. (Fowl No. 115/33.) × 100.

FIG. 27.—The same, showing periphery of leukæmic nodule and infiltration of liver-tissue by myeloid cells. × 450.

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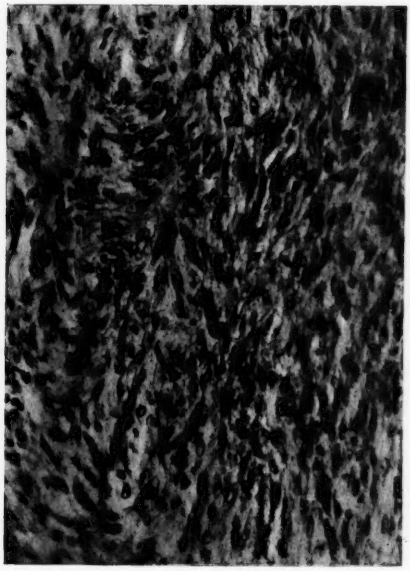

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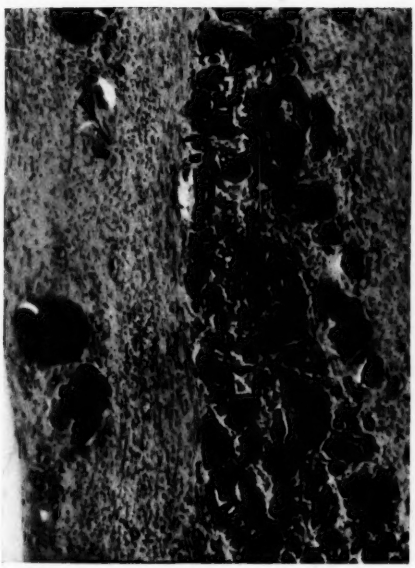

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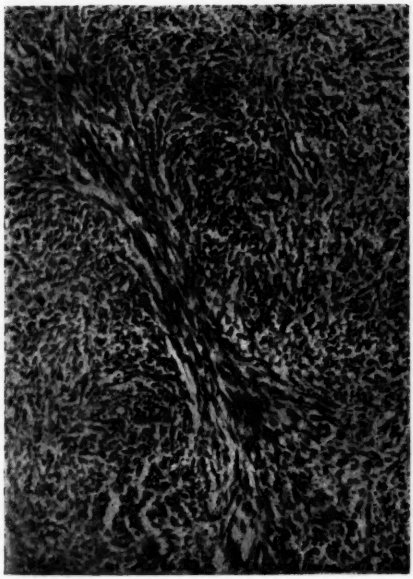

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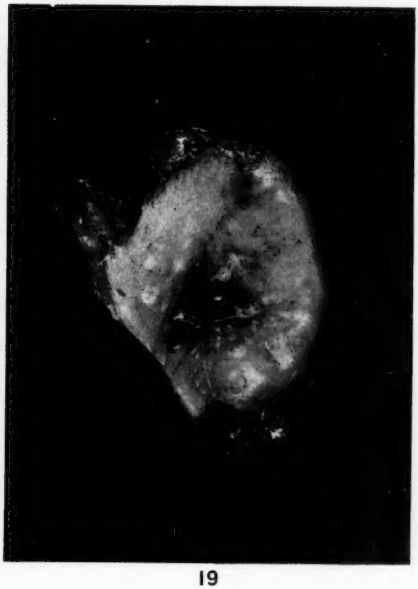

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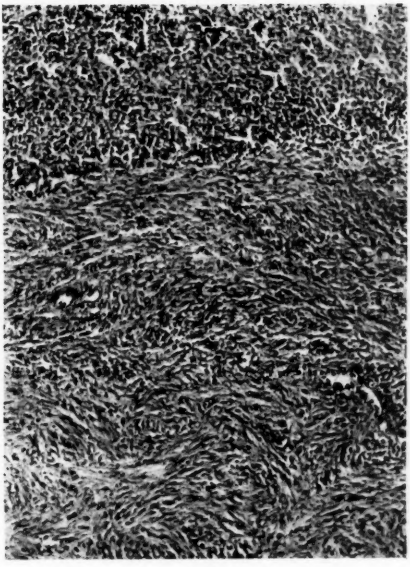

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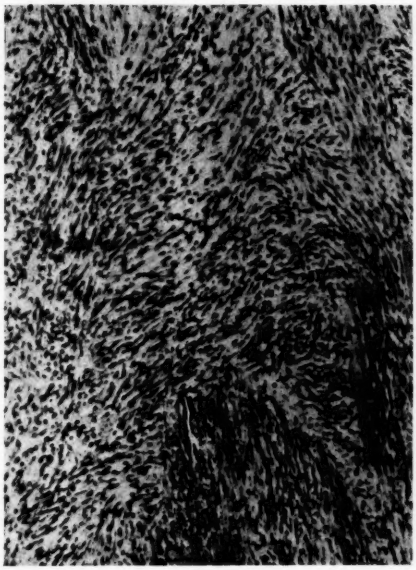

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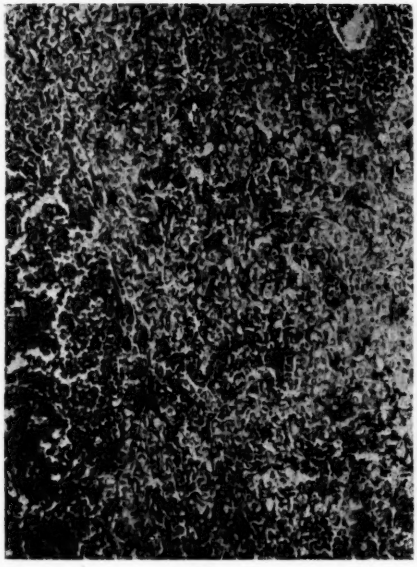

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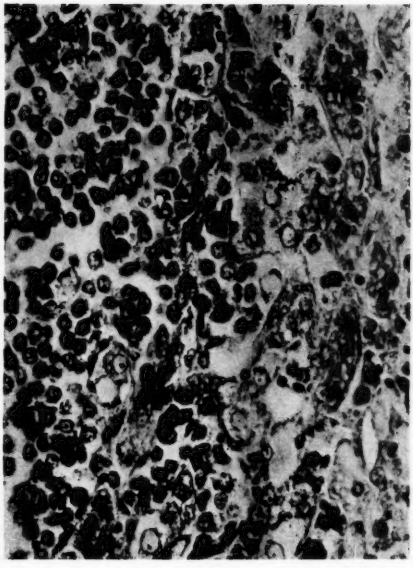

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Tumour 8 (from Fowl No. 332/33, Experiment 5).

Ordinary and trypsinized emulsions of the primary breast tumour were inoculated in the breast-muscles of 6 fowls without result. Three fowls similarly inoculated with emulsions of the liver metastasis also gave no result. Four fowls were inoculated intraperitoneally with emulsions of the liver metastasis. At 219 days a tumour could be palpated at the lower end of the sternum in one fowl. When the fowl was killed at 221 days, the tumour was found to have penetrated the sternum from the peritoneal cavity. There was also a tumour 1 in. in diameter attached to the posterior wall of the peritoneal cavity and infiltrating the ribs. The liver was slightly enlarged and mottled throughout with small white nodules. In the lower part of the left lobe there were several larger nodules, up to ½ in. in diameter. The tumours were softer than the usual tar tumours. In the 2nd passage ordinary emulsion of the breast and intraperitoneal tumour were inoculated into the breasts of 6 fowls without result. Intraperitoneal inoculations of the breast tumour in 3 fowls gave no result. Of 3 fowls inoculated intraperitoneally with an emulsion of the intraperitoneal tumour, 2 were negative, and 1 died at 679 days with tumour masses throughout the liver. Parts of the liver were replaced by solid tumour, and the rest of the liver was studded with small growths. The ovary was also infiltrated by growth. In the third passage 6 fowls were inoculated with ordinary and trypsinized emulsion in the right and left breasts. Tumours were produced by all the inoculations within 33 days. The tumour was kept for 8 passages, but failed to grow at the 9th passage.

Filterability.—At the 4th passage a Berkefeld filtrate of a trypsinized emulsion was inoculated in the breasts of 3 fowls. One died at 64 days with a small tumour. The others were negative. At the 5th passage 6 fowls were inoculated with Berkefeld filtrates of ordinary and trypsinized emulsions with no result. At the 6th passage 8 fowls were inoculated in the breast-muscle with a Berkefeld filtrate of a trypsinized emulsion and all inoculations produced palpable tumours within 23 to 40 days, except in one fowl, which died at 24 days.

Histology.—The 1st passage tumour consisted of densely-packed leukæmic cells, with a very fine stroma (Figs. 12 and 13). In parts the cells were undifferentiated, and in other parts they were more differentiated, showing eosinophil granules in the cytoplasm. The heart blood contained large numbers of myeloblasts and myelocytes. The liver, spleen and ovary showed extensive myeloid infiltration. No evidence of sarcoma could be found. The 2nd passage tumour was composed of myeloid and connective-tissue elements in intimate association (Figs. 14 and 15). The connective tissue was well-differentiated and provided a stroma for the myeloid cells, which were more primitive than those found in the 1st passage. The ovarian tumour contained fewer myeloid cells, and presented the appearance of a fibrosarcoma with pockets of primitive myeloid cells. At the 3rd passage the tumours were firm and fibrous, and consisted of closely-packed bundles of spindle-cells with an abundant network of collagenous fibres, and were indistinguishable from the primary tumour. There were myxomatous areas and lymphocytic reaction at the periphery. The myeloid cells had completely disappeared. In the subsequent passages the tumour remained free of leukæmia and maintained its well-differentiated

character, but showed a slightly greater tendency to myxomatous degeneration (Figs. 16 and 17).

Tumour 9 (from Fowl No. 787/34, Experiment 6).

Fourteen fowls were inoculated with ordinary and trypsinized emulsions of the primary tumour in the right and left breasts. Tumours were found in both breasts of 2 fowls at 141 and 203 days. The tumours were very firm and infiltrated the muscle. At the 2nd passage tumours from a trypsinized emulsion of the 141 days' tumour were palpable at 67 days in 2 of 8 fowls. Tumours from a trypsinized emulsion of the 203 days' tumour were palpable at 34 days in 2 of 16 fowls. All subsequent passages were done with tumour emulsions or filtrates. Propagation was discontinued at the 42nd passage and a tumour desiccate stored.

Filterability.—At the 3rd passage 4 fowls were inoculated with the Berkefeld filtrates at pH6 in the right breast and at pH9 in the left breast. Six fowls were also inoculated with a Berkefeld filtrate treated with trypsin. All inoculations were negative. At the 4th passage 4 fowls were inoculated with Berkefeld filtrates of ordinary and trypsinized emulsions in the right and left breasts. Of the trypsin-treated inocula, two gave palpable tumours at 16 days, and one at 30 days. The ordinary inocula gave only one small nodule, discovered at autopsy at 16 days. A portion of the trypsinized Berkefeld filtrate was spun in the high-speed centrifuge for 30 minutes at 40,000 r.p.m. The supernatant fluid gave no tumour, and the deposit produced a palpable tumour in 16 days. At the 5th passage two tumours were produced with a trypsinized Berkefeld filtrate and no tumour with an ordinary Berkefeld filtrate in 4 fowls.

Histology.—The 1st passage tumours were fibrosarcomata similar to the primary tumour. At the 2nd passage the tumours became less differentiated, and consisted of loosely-packed, long spindle-cells, with considerable myxomatous degeneration (Figs. 24 and 25). It has retained this appearance throughout its propagation, except for a greater tendency to myxomatous degeneration in the later passages. Older tumours are firmer and more compact and resemble the primary tumour more closely.

Tumour 10 (from Fowl No. 699/37, Experiment 7).

The tumour from the right breast failed to grow after transplantation into the right breast of 16 fowls, 6 of which were inoculated with minced tissue and 10 with trypsinized emulsion. The left breast tumour inoculated in the same manner in the left breasts of the same 16 fowls was successful in 4 of the fowls inoculated with minced tissue. The other inoculations failed. The tumour was successfully propagated for only 6 passages with minced tissue. Five attempts were made to transmit the tumour with dilute emulsions, but they were unsuccessful. A sand and paper-pulp filtrate of the 5th passage also failed to take. Tumour 10 maintained its original characters throughout its propagation, being firm, fibrous and, on microscopical examination, well differentiated.

DISCUSSION.

Difficulty of Propagation.

These later experiments have provided four additional tar-induced tumours in the fowl, which have been propagated in series. Two of these tumours have been successfully filtered. Thus, including the earlier experiments, there have been obtained in all 9 propagated tumours, and of these 5 have been filterable. The variability of the biological behaviour of these tumours may help to explain the discrepancy between our findings and those of other workers, particularly in relation to filterability. Considering the small number of chemically-induced tumours which have been successfully propagated in series by other workers, it is not surprising that they have only obtained tumours which they claim to be non-filterable. We have also found that tumours can be readily induced with 1 : 2 : 5 : 6-dibenzanthracene, but these were hard and fibrous tumours, and we had no success in propagating them. In one experiment 9 Plymouth Rock fowls were given four fortnightly injections of 0·5 c.c. of 0·4 per cent. 1 : 2 : 5 : 6-dibenzanthracene in lard in the right breast. Fifty-six days after the first injection there were only 3 survivors, and tumours were found in all 3 at 62, 62 and 90 days. It would appear, therefore, that dibenzanthracene tumours in fowls are less virulent than tar tumours, and for this reason we have continued to use tar as the inducing agent.

A factor which perhaps has contributed largely to the success of our experiments has been the technique employed in the transplantation of the tar-induced tumours. It would appear essential, in the first instance, to use the tumour when it is young and actively-growing, whilst the number of fowls (12 to 20) must be adequate to make sure of having susceptible fowls in each batch (Fig. 3). Then it is essential that minced tumour emulsions be used for the first passages as well as the more dilute tumour emulsions. It is also important to use dilute tumour emulsions for the routine transmission of these tumours, and particularly after treatment with trypsin (Baker and McIntosh, 1927). With the exceptions of Tumour 10 and Tumour 7 in its later passages, all the tumours have been propagated with dilute emulsions. With trypsinized emulsions the tumours usually appear much earlier and grow more vigorously. It is usual that after several passages by inoculations with emulsions the percentage of successful emulsion inoculations increases considerably. A tumour, however, may lose this habit, as is shown by Tumour 7, which, after transmission by emulsions for 10 passages, could then be maintained only by minced tissue inoculations. In our experience a tumour which will not grow from injections of tumour emulsion will not yield an active Berkefeld filtrate. Our experiments would show that these fowl tumours can be trained to require fewer cells for transmission, and that the filterable agent thus takes a more active part in the transmission of the tumour. This would apply particularly when trypsinized emulsions are used, as the action of the trypsin would discourage cellular transmission. We have observed a similar phenomenon in the Rous sarcoma No. 1, where repeated passages by filtrates has greatly increased the virulence of Berkefeld filtrates, while a similar rapid passage by tumour emulsions had no such effect.

Association of Fowl Sarcoma with Leukæmia.

A good deal of experimental work has been done on the association of fowl tumours and leukæmia. Furth (1931*a*, 1931*b*), Oberling and Guérin (1933), Rothe Meyer and Engelbreth-Holm (1933) and Stubbs and Furth (1935) have all described fowl tumour strains which produce both sarcoma and leukæmia. None of these authors has been able to isolate agents producing the two conditions separately, and all have concluded that a single agent may produce both conditions. In our first series of experiments (McIntosh, 1933) a similar feature was recorded, but all our evidence goes to show that two viruses are present, and that the result of passage depends on the relative virulence of the two agents and the site of inoculation. It has already been mentioned that Tumour 8 in the 1st passage gave only leukæmic tumours. In the 2nd passage there were both leukæmic and connective-tissue elements, and in subsequent passages sarcoma only. Tumour 3 in a substrain showed a similar sequence of events, as is shown in Fig. 1, where for three passages the sarcoma was replaced by leukæmia (Figs. 26 and 27). The sarcoma emerging from the leukæmic passages was essentially the same as the original tumour both in behaviour and in appearance, and in neither tumour did leukæmia reappear in subsequent passages. The tumour agent can therefore be carried over in a leukæmic condition without producing a sarcomatous tumour.

Collections of lymphocytes, usually at the periphery of these tar-induced tumours, were frequently found. A diffuse eosinophil infiltration is also a frequent finding, more marked in some than in others. We regard both these conditions as inflammatory reactions, not to be confused with leukæmia.

Filterability.

It has been suggested that the filterable tar-induced tumours may possibly be of spontaneous origin. In the first place, during the last twelve years there have been only three spontaneous tumours in a total of about 8000 fowls. This extremely low incidence is explained by the fact that we have used in our experiments chickens of only about three months. On the other hand, the incidence of tumours in the fowls inoculated with tar has been about 40 per cent.

The question of the association of leukæmia and tumours in fowls has also led to a similar criticism (Peacock, 1935*b*). It is true that in the earlier experiments leukæmia was found in some of our tumour-bearing fowls. We have already dealt with this question, and have produced evidence to show that the agents of tumour and leukæmia are separate entities. Furthermore, in our later experiments we worked with a strain of fowls which has only on the rarest occasions ever shown evidence of leukæmia, with the result that none of our later tar-induced tumours has been associated with leukæmia except in a passage experiment. Leukæmia, therefore, does not play an essential part in the production of these tar-induced filterable tumours.

Again the fact that these earlier tumours were not always found exactly at the site of the tar injection has also been urged as a criticism of the earlier findings (Andrewes, 1936). The following considerations show that this is not a legitimate criticism. Tar can be carried some distance from the site of the

inoculation and form a tumour, or a metastasis may form from an unrecognized primary tumour. More important, however, is the relative incidence of tumours in our normal fowls and in the injected fowls, to which reference has already been made. Furthermore, all our later tumours were found in the breast-muscle with tar deposits in their centres.

Moreover, the tar-induced tumours can all be distinguished from each other and from other tumours by their consistency, histological characters and biological behaviour. The two tumours which most closely resemble the Rous sarcoma No. 1, Tumours 5 and 9, grow much more slowly than the Rous tumour. The average interval between passages in Tumours 5 and 9 is 25 days, whereas the Rous tumour in our hands has had to be transmitted every 19 days on an average during the past five years.

The filterable agents in these tar-induced tumours are similar in their behaviour to the agent of the Rous sarcoma No. 1. They can be sedimented in the high-speed centrifuge at approximately the same gravimetric pull, they survive repeated freezing and thawing, they withstand desiccation, and tumour filtrates are increased in activity by treatment with trypsin.

Other workers have brought forward direct or indirect evidence in support of the view that chemically-induced tumours in fowls are virus tumours. Des Ligneris (1935, 1936) produced a tumour in the fowl by the inoculation of tissue cultures of chick fibroblasts that had been treated with a very small quantity of 1 : 2 : 5 : 6-dibenzanthracene for four weeks. The tumour was successfully transplanted, and sand and paper-pulp filtrates became active at the fourth generation. By cross-immunity experiments he showed that this tumour was distinct from the Rous sarcoma No. 1. Carr and Amies (1938) in a recent investigation have also shown that the des Ligneris tumour and the Rous sarcoma are distinct tumours, although closely related.

Tumours have been induced in fowls with tar or 1 : 2 : 5 : 6-dibenzanthracene by Murphy and Landsteiner (1925), Peacock (1933, 1935*a*), Mellanby (1934), and Gye and Purdy, but none of these tumours has proved to be filterable in the sense of being transmissible by means of Berkefeld filtrates. Andrewes and Foulds, however, have brought forward evidence that two of the above non-filterable tumours have antigenic properties closely related to the virus of Rous sarcoma No. 1. Andrewes (1936) found that pheasants inoculated with the Sheffield tar sarcoma developed antibodies which would neutralize the virus of the Rous sarcoma. Foulds (1937) gave rabbits repeated inoculations of extracts and Berkefeld filtrates of the dibenzanthracene tumour RFD2. The rabbit sera neutralized the virus of the Rous sarcoma after both extract and Berkefeld filtrate inoculations. Both these authors concluded that a virus was present in the tumours. We have also, on several occasions, obtained successful tumour passages of the Sheffield dibenzanthracene tumour (Mellanby, 1934) by means of sand and paper-pulp filtrates, although we have never yet been successful when a Berkefeld filter was used. Our experience has shown that only filterable tumours can be successfully transmitted by means of sand and paper-pulp filtrates, and the difficulty of obtaining an active Berkefeld filtrate from this tumour is probably due to the larger size of the virus.

Mellanby (1933, 1934) found that tissue from a filterable tumour (the Rous sarcoma) would grow after practically all oxidation and glycolytic action in

the tumour-cells were destroyed by chemical treatment, whereas tissue from a chemically induced non-filterable tumour would not grow even after much less chemical treatment. He concluded that his experiments supported the view that filterable virus tumours and chemically induced tumours are widely different in ætiology. We, on the other hand, would interpret his findings as showing that a virus can withstand chemical treatment sufficient to damage tumour-cells irreparably and that if the virus is in an inactive form no tumour will result when the tumour-cells are destroyed. As was found by Andrewes and Foulds, an extract from such a tumour is antigenic, a result which one would expect from the presence of an inactive virus.

CONCLUSIONS.

The most important conclusion that can be drawn from our work is that an active virus can be demonstrated in a large proportion of chemically-induced connective-tissue tumours in the fowl. In a few of these tumours Berkefeld filtrates are consistently inactive—a condition which may be present for considerable periods in admittedly filterable tumours, such as the Rous sarcoma No. 1 (Gye and Andrewes, 1926). This may be due to variations in the relation of the virus to the tumour-cells to a decrease in virulence, to the presence of an inhibitory substance in the filtrate, or to the virus being too large to pass through a Berkefeld filter. The virus nature of these tumours is further supported by the demonstration of antigenic properties by Andrewes and Foulds. In addition, Parsons (1936, 1938) has reported the successful filtration of a chemically-induced tumour of the mouse, Mal. sarcoma 1, a tumour associated with leukæmic blood-changes.

Although there is still much work to be done with regard to the effects of carcinogenic agents on the tissues of the fowl, it would appear from the arguments we have brought forward, both direct and indirect, that tar-induced sarcomas of the fowl are virus tumours, and therefore presumably filterable. This being the case, carcinogenic agents (tar, 1 : 2 : 5 : 6-dibenzanthracene) can only be factors in the induction of these tumours, and play no part in the maintenance of the subsequent malignant process.

SUMMARY.

Four new tar-induced tumours have been propagated in series and of these two have been transmitted repeatedly by Berkefeld filtrates.

One of the non-filterable tumours could be passed by means of emulsions and the other by means of minced tissues only.

We have also succeeded in passing by means of sand and paper-pulp filtrates another fowl tumour (the Sheffield dibenzanthracene sarcoma), a tumour which is non-filterable in the sense that it is not transmissible by means of Berkefeld filtrates.

Suggestions are given in explanation of the variation in biological behaviour of these induced tumours, and in particular the variation in their filterability.

The earlier observation that tar can induce filterable tumours has been confirmed.

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THE ANTIGENIC SURFACE OF SMOOTH *BRUCELLA ABORTUS* AND *MELITENSIS*.

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THE antigenic surface of smooth *Brucella abortus* and *melitensis* is such that antisera can be exhausted of all agglutinins by large doses of heterologous bacterial suspensions; absorption by smaller doses leaves agglutinins effective only against the homologous suspension. These facts point to the existence of two antigens, A and M, on each bacterium, A predominating in *abortus* and M in *melitensis* (Wilson and Miles, 1932; see also refs. Topley and Wilson, 1936). Constant-antigen titrations of Brucella suspensions against falling dilutions of antiserum exhibit an optimal zone of rapid particulation flanked by prozone of retardation or inhibition in strong antiserum, and a "postzone" of retardation in weaker antiserum (Miles, 1933). This paper reports a further analysis of the particulation times of Brucella agglutinating systems. The same titration technique has been used, and in the main the study has been confined to typical smooth strains of *Br. melitensis* (strain Younowitch 6) and *Br. abortus* (strain Kristensen 25), and their respective antisera.

THE CONSTANT-ANTIGEN OPTIMUM AND PROZONE.

The collision rate of the individual bacteria is constant in a standard bacterial suspension, so that the differences in the rate of flocculation must be dependent on the rate of sensitization. In the postzone, the dilution of antibody results in a slow rate of sensitization of the bacteria. The mechanism of retardation in the prozone is less clear; to explain it, Ehrlich postulated the existence of agglutinoids (see Shibley, 1929), antibodies having a greater affinity for antigen, but less flocculating power, than agglutinin. There is no direct evidence for the existence of agglutinoid in the Brucella sera studied. Moreover, the following experiment, typical of three, shows that the agglutinin firmly adsorbed in the prozone has the same power of sensitizing the bacteria to the action of salt as that adsorbed at the optimum.

Suspensions of *Br. abortus* in distilled water were heated at 56° C. for one hour with salt-free antiserum in two dilutions, known to produce total prozone inhibition and optimal agglutination respectively in 0·85 per cent. saline titrations. The two sensitized but unagglutinated suspensions were thrice centrifuged and washed in distilled water, and diluted in distilled water to an opacity of 2×10^9/c.c. The addition of an equal volume of $2 \times 0{\cdot}85$ per cent. saline produced standard agglutination of both suspensions in the same time. Antibody was detectable in the first washings of the prozone-sensitized bacteria. This does not affect the validity of the experiment, for agglutinoid, having by definition a greater affinity for antigen than agglutinin, should have remained on the bacteria when agglutinin was removed, and rendered them less salt-sensitive than those optimally sensitized.

In some agglutination systems, the optimum is shifted into lower serum concentration by lowering the salt concentration (*cf.* Duncan, 1937; Heidelberger and Kabat, 1938), and it might be objected that the concentration of antibody, which was optimum in 0·85 per cent. saline, was in excess in distilled water, and both suspensions may have been sensitized partly by agglutinoid. Experiments with Brucella systems show that the shift of the optimum with a reduction of salt concentration from the 0·85 per cent. level is small, and approximately proportional to the log. salt concentration. With the system used the 0·05 per cent. salt optimum was only 1·4 times the 0·85 per cent optimum, and for the purposes of the above experiment, in which the antibody excess mixture was 16 times as strong, the two optima can be considered substantially the same.

The optimum may be regarded as the point of maximum sensitization of the antigenic surface by antibody, and retardation in stronger antiserum as due to excess of the same kind of antibody. Both optimum agglutination and its retardation in antibody excess may be interpreted in the light of one of two hypotheses. Eagle (1930) elaborated the view that at maximum sensitization there is a hydrophobe layer of antibody over the antigenic surface. Retardation in antibody excess occurred only once in the systems he studied (Eagle, 1932, Fig. 6) and was not considered in his papers. Nevertheless an hypothesis of prozone formation may be derived from Eagle's hypothesis. At maximum sensitization the hydrophobic nature of the combined antibody is due to a distortion or reorientation of the globulin molecule consequent upon the combination of its receptors with determinant groups on the antigenic surface; the antibody molecule combines with a relatively large antigenic area. In mixtures containing antibody in excess of the amount producing maximum sensitization, the reorientation, postulated by Eagle, of one antibody molecule at the antigenic surface would be inhibited to a certain extent by the presence of others occupying adjacent antigenic groups. With every increase of antibody concentration the transformation from hydrophile to hydrophobe

would be progressively inhibited, until a limit was reached when so few receptors per antibody molecule were utilized, that there was no detectable alteration in the hydrophile state of the antibody. According to Marrack (1934), bacteria are agglutinated because each antibody particle with more than one receptor is capable of combining with the antigenic groups on two (or perhaps more) bacteria. Maximum sensitization would, in this case, occur when about half the antigenic surface of each bacterium had combined with antibody, leaving half free for combination with antibody already attached to other bacteria; and the resulting agglutinated mass is a " lattice " of antibody and bacteria. If enough antibody was added to cover all the antigenic surfaces in the first stage of the reaction, no such linkage between bacteria would be possible, and inhibition would occur. At the Brucella optima the agglutinated bacteria have been found to adsorb all the antibody present, as would be expected from either hypothesis; antibody was not found in the supernatant fluid after absorption until two to four times the optimal concentration of antibody was used, a finding numerically in accordance with the consequences of Marrack's lattice hypothesis. It is equally compatible with Eagle's views; at the optimum, the number of receptors on each antibody molecule is sufficiently large to produce maximum reorientation of the antibody; if fewer receptors per molecule are involved, the reorientation and consequently the salt sensitization is less, but the antibody present is, nevertheless, completely absorbed by the given antigenic surface. In stronger antiserum, the amount of antibody absorbed was found to be proportional to the antibody excess. For example, in Fig. 10, Col. 3, 85 per cent. is absorbed from four times, and 40 per cent. from sixteen times the optimal antibody concentration (see also Figs. 6–11, and Duncan, 1934, p. 26), *i. e.* the total amount of antibody adsorbable by the suspension from excess antibody is not uniquely determined by the area of antigenic surface exposed, but depends on antibody concentration. The amounted absorbed at maximum saturation has not been determined for Brucella suspensions, but in Type III pneumococcus systems, it is not simply proportional to the absorbing dose of cocci (Heidelberger and Kabat, 1937).

Optima, being independent of absolute measures of agglutination velocity, are peculiarly suitable for determining the simple antibody : antigenic-surface ratio, since the time factor needs no standardization. Of the two available optima, the constant-antigen and the constant-antibody, the latter is too complex for easy analysis, being the point at which, with increasing concentration of bacterial suspension, the decrease in velocity due to decreasing sensitization is balanced by the increase in collision rate of the bacteria. At the constant-antigen optimum, on the other hand (since in a constant saline concentration the optimal ratio of serum concentration to bacterial concentration is a constant (see Miles, 1933; Duncan, 1932 and 1934)), the optimal flocculation and therefore the maximal sensitization of particles of uniform size and homogeneous antigenic surface, by a solution containing one kind of antibody, is determined by the area of effective surface antigen exposed. The antibody adsorbed bears a linear relationship to the area of antigenic surface, and if $[X]$ is the concentration of antigen X on the total surface of a standard bacterial suspension and $[x]$ the concentration of antibody x, then at the optimum $[X]/[x]$ = constant. It follows that, *if unit antibody is defined as the*

amount covering unit antigen surface at the point of maximum sensitization, then in the optimum tube of a simple system, $[X] = [x]$.

DOUBLE AGGLUTINATION ZONES.

The single zone of Brucella system may be described in terms of its optimum. Occasionally double zones occur in Brucella titrations, and it was suggested (Miles, 1933) that they were due to the separate reactions of A and M antigens with their respective antibodies. But in the agglutination of particles bearing two or more antigens by a solution of two or more antibodies, neither the optimum nor the 100 per cent. adsorption concentration is reliable as a measure of antigen or antibody. In certain precipitin titrations, the reaction of two separate antigen-antibody systems produces two separate optimal zones (*e. g.* Dean, Taylor and Adair, 1935 ; Goldsworthy and Rudd, 1935). The occurrence of two such recognizable zones in analogous agglutination titrations is less likely, since the same particle bears the two antigens and is sensitized which ever antigen is sensitized. Assuming the $[X] = [x]$ relationship at the sensitization maximum, the factors influencing the formation of double zones are : (1) The distribution of A and M on the bacterial surfaces ; (2) the proportion of the corresponding antibodies α and μ in the antisera ; this is dependent on (3) the antigenicity of A and M in the immunizing suspension and (4) the mode of immunization of the rabbit used ; (5) the rate of sensitization of A and M in the test-tube, and the relative strengths of A-α and M-μ bonds, depending on the avidity of α and μ. With regard to (3) and (5), we may assume A and M equally antigenic, and the α and μ receptors equally avid ; these assumptions are justified by the similarity of homologous precipitin and agglutinin titres produced in response to injections of *abortus* and *melitensis* and by the similarity of agglutination velocity curves of smooth *abortus* and *melitensis* suspensions (see *e. g.* Figs. 6–11).

The relative amounts of the two antigens.—With regard to (1), some measure of the proportions of A and M on the antigenic surface may be deduced from the following considerations : A monospecific serum (see Wilson and Miles, 1932 ; Wilson 1933), *e. g.* anti-*melitensis*, agglutinates *Br. melitensis* only, in virtue of the its μ antibody content. The M antigens on *abortus* organisms must therefore be insufficient in amount or distribution for the production of the agglutination, even if they are fully sensitized. The homologous antiserum concentration that maximally sensitizes a 10^9/c.c. suspension of *Br. abortus* is about 16 times the end-titre concentration. The velocity of optimal agglutination is not greatly reduced until the antiserum is diluted 4-fold. In this concentration, 10^9/c.c. is the optimal suspension for a constant-antiserum titration (Miles, 1933). In lower concentrations the velocity falls off rapidly, presumably because the available antibody cannot sensitize more than a small proportion of the available antigenic surface ; and the serum is inactive if further diluted 4-fold, *i. e.* if the optimal antibody concentration is reduced over 16-fold, the number of antigenic groups occupied is insufficient to produce agglutination. Alternatively it can be argued that if the number of groups was reduced over 16-fold, their maximal sensitization would be insufficient to agglutinate the bacterium. In an *abortus* A + M, we may

therefore expect the M surface to be of the order of 1/16 or less of the A surface, so that, in the presence of μ antibodies only, *abortus* is not agglutinated.

This ratio may be deduced more directly from the results of the following experiments. It was found that monospecific agglutinins were absorbed by heterologous as well as by homologous suspensions. To avoid as far as possible the effect of high concentrations of antibody on the amount of antibody absorbed, solutions containing a final concentration of two monospecific agglutinin doses were absorbed with graded doses of organisms for 4 hours at 37° C. Unit dose is the minimal amount of antibody producing standard agglutination of a 10^9/c.c. suspension of smooth organisms in 4 hours at 56° C. A gross difference in the sensitivity of *melitensis* and *abortus* suspensions to agglutinin would invalidate this method of measuring monospecific sera, for the *a* and μ doses might represent very different amounts of antibody. *Br. abortus* and *melitensis* are liable to S—→R variation, with a consequent loss of S antiserum sensitivity. It was found, however, that a drop in anti-S end-titre did not occur until there was a small exposure of R antigen on the bacterial surface, and a marked loss of total smooth SSS content. Up to this point the end-titre is independent of the absolute amount of antigenic surface per bacterium, and agglutination is presumably dependent on the effective sensitization of a certain portion of this surface. Given fully smooth suspensions, it may be assumed, therefore, that unit doses of *a* and μ agglutinins represent comparable amount of *a* and μ antibody. Table I records the minimal total absorbing doses of suspensions. Loiseau is a South African, and Lanyon a recently isolated English strain. Comparing mean doses of homologous and heterologue for each monospecific serum, the *melitensis* surface has one fortieth the absorbing power of the *abortus* surface for the *a* antibodies, and *abortus* one-tenth that of *melitensis* for the μ antibodies. Comparing the doses of each separate species, for homologous and heterologous sera, the ratio of average absorbing doses for *a* and μ antibodies is 1 : 37 for *abortus* and 11 : 1 for *melitensis* suspensions. The inference of ratios of A and M on the bacterial surface from these ratios of doses of suspension is legitimate if (*a*) all the suspensions have about the same amount of effective antigenic surface per 10^6 organisms and (*b*) the amount of antibody taken up by a *minimal* total absorbing dose is proportional to the antigenic surface.

TABLE I.—*The Absorption of Two Agglutinating Doses of Monospecific Sera by Homologous and Heterologous Suspensions.*

Monospecific serum.	Minimum total absorbing dose (millions).				Ratio of mean absorbing doses *abortus* : *melitensis*.
	abortus suspensions.		*melitensis* suspensions.		
	K25.	Lanyon.	Y6.	Loiseau.	
abortus, anti-K25	125	93	4000	5000	1 : 41
melitensis, anti-Y6	3000	5000	300	500	10 : 1
Ratio of mean absorbing doses in *a* and μ sera	1 : 37		11 : 1		

With regard to (*a*) the similarity of the two sets of ratios, 41 : 1 and 1 : 10, 37 : 1 and 1 : 11, excludes the possibility that the divergence in the two species represents merely a difference in amount of effective antigenic surface. With regard to (*b*) a few experiments absorbing 2, 4 and 6 agglutinating doses with a series of suspension dilutions have shown that the minimal total absorbing dose is approximately proportional to the antibody concentration. Nevertheless, a large number of tests of recently isolated strains is required before these ratios can be accepted as measures of the ratio of A to M antigens on the surface. The A : M ratios for Y6 and K25 on the basis of the results in Table I are 1 : 30 and 10 : 1, but for the purpose of quantitative analysis, the average values of 1 : 20 and 20 : 1 will be adopted as a provisional measure of the ratios in *Br. melitensis* and *abortus*. The assumption of such a reciprocal similarity in the ratios does not affect the conclusions reached in this paper, for they are equally valid on the assumption that the proportion of minor antigen varies from species to species.

It is probable that this surface relationship also holds for the *total* amounts of the two antigens in the whole bacterium, since the minimal homologous and heterologous absorbing doses for monospecific sera, of partially rough organisms that contain less than 10 per cent. of specific soluble substances (SSS) extractable from the fully smooth suspensions, and of smooth suspensions from which over 50 per cent. of SSS has been extracted, are in approximately the same ratio as those for the smooth suspensions.

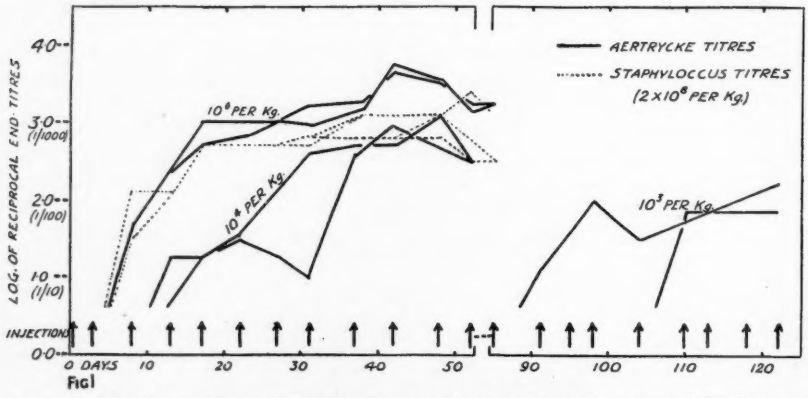


FIG. 1.—Agglutinin production in 6 rabbits after repeated injections of a mixture of *Staph. aureus* (dotted lines) and small quantities of *Bact. aertrycke* (continuous lines).

The relative amounts of the two antibodies.—With regard to (2) and (4), the proportion of α and μ in the antisera will depend on the response of the rabbit to A and M. The dependence of antibody proportions on the time factor is illustrated in Fig. 1 ; in which is recorded the agglutinin response of rabbits to multiple injections of a mixture of antigens, *Staph. aureus* as the major and *Bact. aertrycke* as the minor antigen.

The *Staph. aureus* was heat-killed and washed in saline; the *Bact. aertrycke*, a smooth "H" strain, was formol-killed in the cold, and the exact content of a suspension of about 7×10^8/c.c. was determined by Helber chamber counts. Mixtures for injection were made containing a standard 200×10^6 staphylococci per c.c. and 10^7, 10^6, 10^5 and 10^4 *aertrycke* per c.c. Young rabbits, 1·7 to 2·2 kg. in weight, were tested for freedom from natural staphylococcus or *aertrycke* agglutinins (6 of 32 normal healthy breeding stock had titres of 1 : 4 or 1 : 8 for *aertrycke*) and four pairs injected at 4 to 5-day intervals with 1 c.c. per kg. body-weight. Samples of serum were taken immediately before each injection.

The response to the flagellar antigen of 10^7, 10^6 and 10^5 *aertrycke* per kg. body-weight was similar. Agglutinins appeared after 2–3 injections, and increased at the same rate as the staphylococcus agglutinins. In spite of the greater dosage of staphylococci, the superior antigenicity of *aertrycke* and the sensitivity of H bacteria to agglutination results in curves that follow the

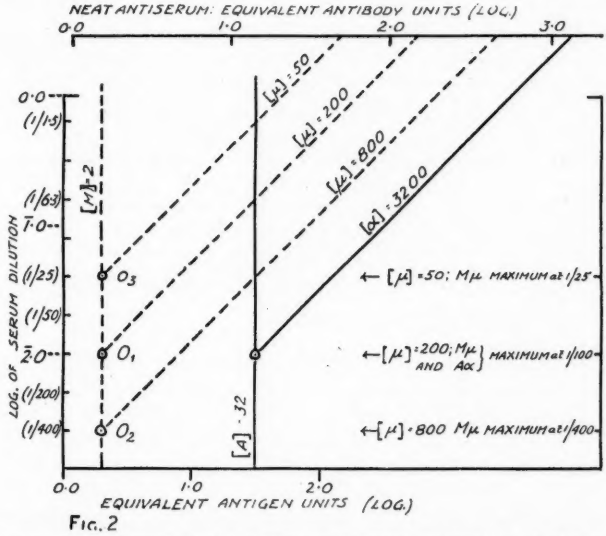


FIG. 2.

FIG. 2.—Relations of the hypothetical sensitization maxima produced in an *abortus* suspension of surface 32M, 2A, by *abortus* antisera of various $[\alpha]/[\mu]$ ratios.

staphylococcus curves closely. Many repeated doses of 10^4 *aertrycke* are required to produce detectable agglutinins, and the two curves lag behind the staphylococcus curves. Two more rabbits were given repeated doses of 10^3 *aertrycke* per kg. body-weight. Only after 91 and 110 days respectively did specific agglutinins appear in the serum. It is of interest that 3 doses of 10^4 (30,000 bacteria) were required to produce the same minimum response as 15 and 18 doses of 10^3 (15,000–18,000). This figure, $20–30 \times 10^3$ is about one quarter of the minimum single dose (10^5) of *Bact. paratyphosum* B required to produce detectable H agglutininins in the rabbit, as determined by Topley (1930).

The representative curves in Fig. 1 illustrate the increasing disparity of the ratios between two antigens and two antibodies that occur with repeated injections of an antigenic mixture; and, when both antibody-responses reach their maxima, the production of antibody mixture bearing no quantitative relation to proportion of the antigens present in the injected material.

Hypothetical conditions for the formation of double zones.

Adopting for arithmetical convenience A/M ratios of 16 : 1 and 1 : 16, the conditions for the formation of double zones may be illustrated as follows :

Suppose the area of A on *Br. abortus* is 16 times that of M. The standard suspension of 10^9/c.c. may be represented 32A, 2M. At an early stage in immunization, the antibody response may be proportional to the antigenic distribution, *i. e.* $[\alpha]/[\mu] = [A]/[M]$. But after repeated injections, the response to A reaches a maximum while the output of μ is increasing. $[\mu]$ approaches $[\alpha]$ and $[\alpha]/[\mu] < [A]/[M]$. Similarly, in the later stages of a *melitensis* immunization, if *melitensis* is 32M, 2A, $[\mu]/[\alpha] < [M]/[A]$. In general, the minor antigen stimulates the proportionately greater antibody response, and in both antisera, $[\mu]$ and $[\alpha]$ will tend to equality.

Returning to the *abortus* system, where $[A]/[M] = 16$, there are three possible relative positions of the sensitizing maxima, depending on the relative proportions of α and μ in the antiserum (Fig. 2):

(1) In the early stages of immunization, if $[A]/[M] = [\alpha]/[\mu]$, the A and M maxima are coincident ; *i. e.* if the concentrations are expressed in the units defined above for any antigen $[X]$ and its antibody $[x]$, $[\alpha]/[A] = [\mu]/[M]$. This is shown at 0_1 ; the antiserum is 3200α, 200μ, and two maxima are at 1 : 100.

(2) If $[\alpha]/[A] < [\mu]/[M]$, the M maximum is lower (in the lower serum concentration) than the A maximum. The antiserum is 3200α, 800μ ; the M maximum, 0_2, is at 1 : 400.

(3) If $[\alpha]/[A] > [\mu]/[M]$, the A maximum is lower. In Fig. 2 the antiserum is 3200α, 50μ, and the M maximum (0_3) is at 1 : 25.

If the A and M perpendiculars are interchanged, Fig. 2 represents the reaction of a *melitensis* strain 32M, 2A with the *abortus* serum. The $[\alpha] = 3200$ diagonal cuts $[A] = 2$ at 1 : 1600, and the $[\mu] = 800$ cuts $[M] = 32$ at 1 : 25. Even with $[\mu] = 3200$, the Mμ maximum will be in 1 : 100 only. In such a heterologous system, the Mμ maximum could not occur in as great a serum dilution as the Aα maximum unless $[\alpha]/[\mu] > 1/16$, a predominance of μ antibodies highly unlikely to occur in an *abortus* serum.

In general, the maximum in the lower serum concentration (high dilution) is due to the antigen proportionately over-represented by antibody. In the homologous *abortus* system, it will be due to M, and in the homologous *melitensis*, to A.

The optimum and therefore the sensitization maximum even of a single antigen system may be broad. For example, using a standard, approximately monodisperse suspension of collodion particles, 2–5μ in diameter, with an adsorbed layer of horse-serum albumin (prepared as Jones, 1928), it was found that the most rapid particulation optimum was spread over an 8-fold dilution range of anti-albumin serum. In mixed systems broader zones may be expected, and, in addition, the sensitization maxima will not necessarily appear as particulation optima. Suppose the A maximum at 1 : 25 and the M at 1 : 100. In the intermediate serum dilution, 1 : 50, the retardations due to insufficient α and excess of μ are small, and the additive effect of the two near-maximal sensitzations may produce agglutination more rapidly than at 1 : 25 or 1 : 100. The presence of two zones thus merged could be inferred only from the broadness of a main agglutination zone. Rabbit sera taken early in the course of Brucella immunization give relatively sharp single zones of agglutination ; later sera give broad zones (Fig. 3), which may be due to the merging of two or more sensitization maxima. Curve 3 in fact suggests a partial merging of two zones.

Analysis of double-zoning systems.—It is probable that the two zones depend on two specific antigens, since in certain cases a well-defined double zone was

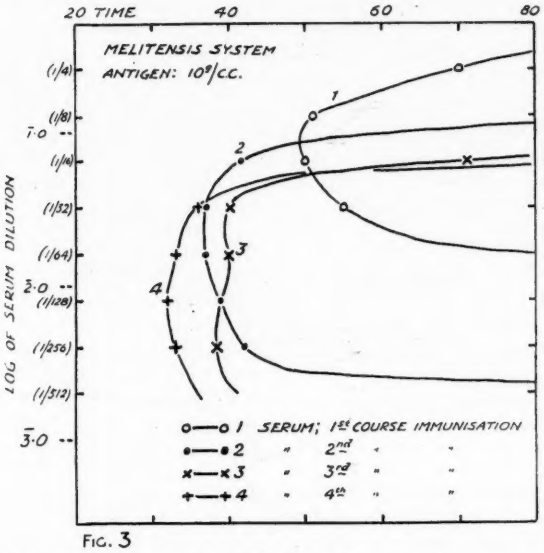


FIG. 3

FIG. 3.—Particulation zones of sera from one rabbit, bled after successive courses of immunization.

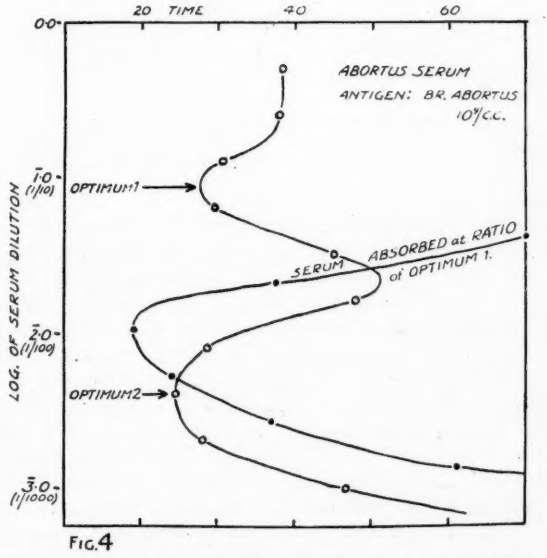


FIG. 4

FIG. 4.—Removal of one optimal particulation zone from a double-zoning *abortus* antiserum by selective absorption.

reduced to a single zone by absorption at the upper optimum. Fig. 4 represents a double zone produced by *abortus* K25 with its homologous antiserums. The upper optimum (1) serum concentration was 1 : 12 for a suspension of $2{\cdot}5 \times 10^9$/c.c. Serum 1 : 3 was absorbed by suspension 10×10^9/c.c., and the absorbed serum gave the single-optimum curve, representing the lower optimum (2). Owing to a partial removal of antibody concerned by the absorbing dose used, it occurs in serum more concentrated than the original lower optimum. In view of the possibility of selective absorption of one optimum an attempt was made to reveal 2 optima in sera giving a single wide optimal zone, as in

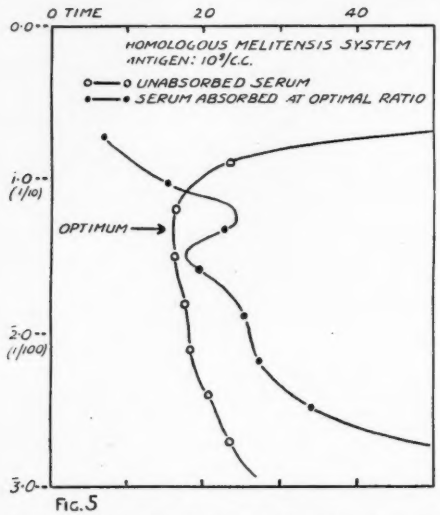


FIG. 5.—Unmasking of double zones in a broad-zoning *melitensis* antiserum by selective absorption.

Fig. 5, which represents a *melitensis* system. The optimum of the large zone of high particulation velocity is in the region of more concentrated serum. A concentrated solution of the antiserum was absorbed by homologous suspension in optimal proportions, and K25 and Y6 suspensions tested against dilutions of the absorbed sera. The figure records the homologous agglutinations times. Although there has been no removal of an upper optimum, the disparity of the [antigen] : [antibody] ratios has been increased and two zones are produced. Similar absorption of an *abortus* serum gave the same result. With the heterologous suspensions, the separation into two zones was less evident ; absorption produced in the main curve an indentation only, corresponding to a lag of a few minutes. A series of experiments on these lines failed to uncover double zones sufficiently separated for subsequent serological analysis. The reactions of the hypothetical A and M antigens with α and μ antibodies appeared to be merged into one main zone ; double zones occurred naturally only in a few systems.

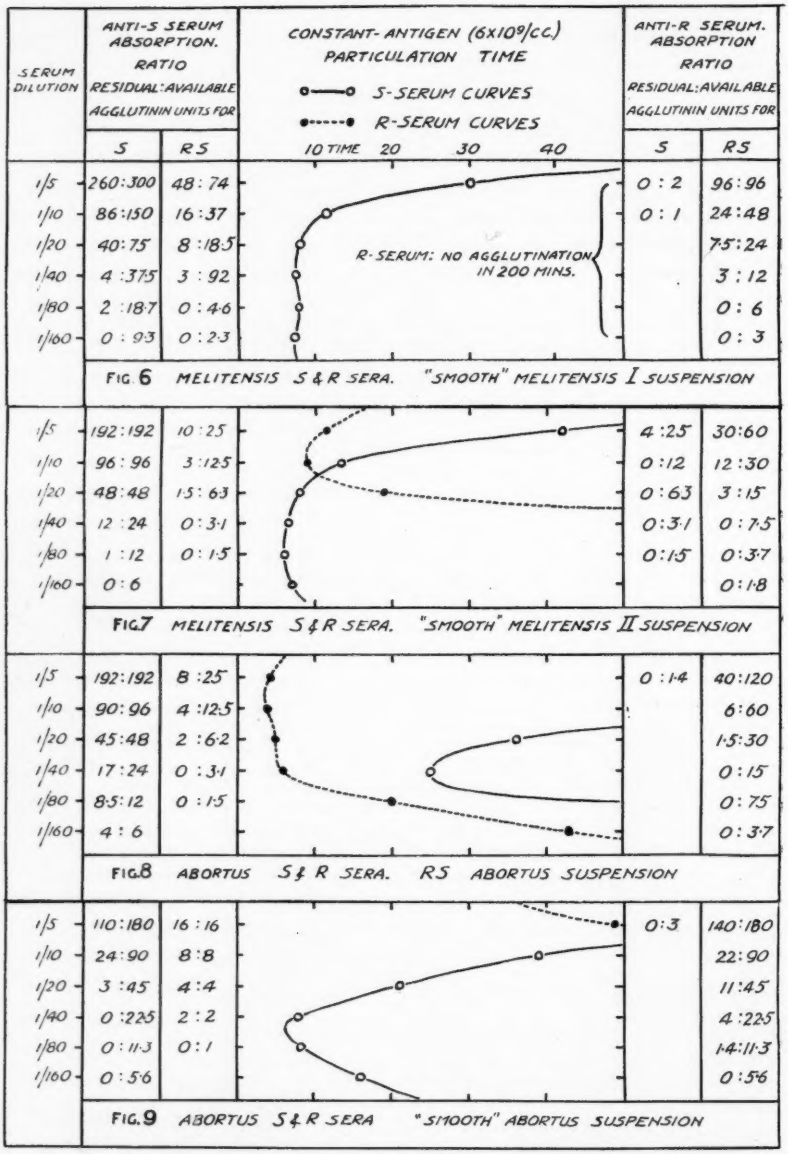
THE AGGLUTINATION OF BRUCELLA VARIANTS.

So far it has been assumed that the antigenic surface of smooth Brucella organisms is completely definable in terms of the A and M antigens. The suspensions of *abortus* and *melitensis* used in the above experiments were agglutinated to full titre by anti-smooth sera, and were stable in 5 per cent. saline at 55° C. and in 0·85 per cent. saline at 100° C. (Pandit and Wilson, 1932). In connection with the harvesting of large amounts of smooth growth for antigen isolation (see Miles and Pirie, 1939) it was found that the amount of specifically precipitable soluble antigen extractable from a given amount of smooth suspension under standard conditions varied considerably with each stock subculture. Careful examination of six stock strains, *Br. melitensis*, Y6 and Loiseau, *Br. abortus*, K25 and Little and *Br. suis*, P1 and P404, revealed in each a continuous series of variants between the fully S form and the manifest R form, which may be represented by S→SR→RS→R. The rough characters of the RS and R form are easily recognizable, but the SR forms, in which the antigen characterizing the smooth form predominates, can be consistently distinguished from S forms only by their total SSS content, an increased titre with R antisera, and occasionally by colony opacity.

Three-day growths of all six strains on nutrient agar yielded three types of colony: (1) a minute dewdrop colony, of fully S forms, that yielded on subculture colonies of the second type; (2) a transparent colony, three times the size of the first, pale cigarette-smoke blue by oblique light; and (3) a semi-transparent colony, dirty yellow by oblique light. All were smooth of surface, entire-edged and easily emulsifiable. The two first grew as a general tubidity in broth, the last as a general tubidity with a slight sediment. The confluent growth from such plates was a mosaic of transparent and semi-transparent patches similar to that figured by Henry (1933*a*) as appearing in *Br. abortus* on glucose-glycerine agar plates. Subcultures from the transparent colonies threw off semi-transparent colonies, but cultures from the semi-transparent never gave transparent. The semi-transparent *abortus* variants were more tolerant of CO_2 lack than the transparent. In general semi-transparent colonies were larger than the transparent, but this was not a constant feature; nor was there any association between the relative sizes of the two variants and the Brucella species, as Henry reports (1933*b*). The semi-transparent evidently correspond to Henry's intermediate type I and to SR and RS forms in the above series. With continuous subcultivation, and selection of the more opaque variants, a slightly granular colony, more difficult to emulsify, is produced, which, though serologically rougher, still contains some smooth soluble substance. Table II shows the direct agglutination end-titres of an SR variant. The R sera were prepared against *Br. paramelitensis* 2062, and a rough *abortus* A.B.O.R. The later bleedings from rabbits so immunized contain antibodies absorbable by smooth SSS, and absorption tests with the earlier bleedings showed that these R sera contained small quantities of S agglutinin, which explains the moderate agglutination of fully S forms by R sera.

The production of SR and even RS variants is not necessarily accompanied by the appearance of a mosaic, though occasionally, when no difference between the opacities of colonies from two cultures could be obtained, the confluent growth from an artificial mixture of the two cultures showed a just perceptible mosaic. Some of the rougher SR variants were feebly agglutinated after 24 hours in 5 per cent. saline, and partially agglutinated after 4 hours at 100° C. in 0·85 per cent. saline. Others were distinguishable from S forms only by the comparatively poor yield of smooth antigen (Miles and Pirie, 1939) from 1/10 to 1/50 the amount from a fully smooth organism, and a slight increase in titre with an anti-rough serum.

Absorption tests confirm the general view that the R antigens of *abortus* and *melitensis* do not differ grossly, for absorbing doses of S organisms that remove all S agglutinins, left R agglutinins that were absorbed by approximately equal minimal doses of R *abortus* and R *melitensis*.


SERUM DILUTION
ANTI-S SERUM ABSORPTION. RATIO RESIDUAL:AVAILABLE AGGLUTININ UNITS FOR
S
RS
CONSTANT-ANTIGEN (6X10⁹/cc.) PARTICULATION TIME
S-SERUM CURVES
R-SERUM CURVES
10 TIME 20 30 40
ANTI-R SERUM. ABSORPTION RATIO RESIDUAL:AVAILABLE AGGLUTININ UNITS FOR
S
RS
1/5 260:300 48:74 0:2 96:96
1/10 86:150 16:37 0:1 24:48
1/20 40:75 8:18·5 7·5:24
1/40 4:37·5 3:92 3:12
1/80 2:18·7 0:4·6 0:6
1/160 0:9·3 0:2·3 0:3
R-SERUM: NO AGGLUTINATION IN 200 MINS.
FIG. 6 MELITENSIS S & R SERA. "SMOOTH" MELITENSIS I SUSPENSION
1/5 192:192 10:25 4:25 30:60
1/10 96:96 3:12·5 0:12 12:30
1/20 48:48 1·5:6·3 0:6·3 3:15
1/40 12:24 0:3·1 0:3·1 0:7·5
1/80 1:12 0:1·5 0:1·5 0:3·7
1/160 0:6 0:1·8
FIG. 7 MELITENSIS S & R SERA. "SMOOTH" MELITENSIS II SUSPENSION
1/5 192:192 8:25 0:1·4 40:120
1/10 90:96 4:12·5 6:60
1/20 45:48 2:6·2 1·5:30
1/40 17:24 0:3·1 0:15
1/80 8·5:12 0:1·5 0:7·5
1/160 4:6 0:3·7
FIG. 8 ABORTUS S & R SERA. RS ABORTUS SUSPENSION
1/5 110:180 16:16 0:3 140:180
1/10 24:90 8:8 22:90
1/20 3:45 4:4 11:45
1/40 0:22·5 2:2 4:22·5
1/80 0:11·3 0:1 1·4:11·3
1/160 0:5·6 0:5·6
FIG. 9 ABORTUS S & R SERA "SMOOTH" ABORTUS SUSPENSION

FIGS. 6–9.—Constant-antigen particulation times of S and SR Brucella suspensions; and ratios of agglutinins left after agglutination, to the initial agglutinin content in each antiserum-suspension mixture. Serum dilutions recorded on a logarithmic scale.

TABLE II.—*Direct Agglutination Titres of S and SR* Br. abortus.

Antigens.	Antisera.		
	Smooth *abortus*.	R *abortus*.	R *melitensis*.
S *abortus*	480	160	240
SR *abortus*	360	480	400

The agglutination reaction of suspensions with various antigenic surfaces is exemplified in Figs. 6–9.

The 55° C. particulation times of 6×10^9/c.c. suspension are plotted against the agglutinin content of each serum dilution used. The agglutinin content is measured in units, which are numerically equal to the reciprocal of the standard agglutination titre with a 10^9/c.c. suspension of the given organism, read after 4 hours at 56° C. The 6×10^9/c.c. agglutination mixtures were held at 55° C. for 2 hours, then at 2° C. for 1 hour, and spun. The supernatant fluid from each, and a control unabsorbed serum similarly heated, were titrated against S and SR suspensions. The ratio of the number of units unabsorbed to those originally present in each dilution is recorded in the columns on the margins of each graph. Partially rough suspensions were chosen in preference to R, since the former are stable in 0·85 per cent. saline, and, as will be seen, the presence of S antigen does not obscure the interpretation of the results.

Figs. 6 and 7 are the curves for two suspensions, I and II, of Y6, made from two apparently smooth single colony cultures, against the same pair of S and R *melitensis* sera. Suspension I is equivalent at 1 : 160 for S and 1 : 80 for RS agglutinins with the S serum and the curve shows faint optima at 1 : 160 and 1 : 40. (The term " equivalent " is used here to mean the maximum concentration of antibody totally absorbed by the suspension, a convenient but loose extension of the " equivalent " of precipitin titrations, which is defined in a titration series as the precipitating mixture in which neither antigen nor antibody excess is in the supernatant fluid. With bacterial antigens, antigen excess is indeterminable in a constant-antigen titration.)

From more concentrated antibody solutions, the suspension takes out RS agglutinins more effectively than the S. With the R serum there was no agglutination in 100 minutes, but RS agglutinins were nevertheless absorbed, and the suspension is equivalent for RS at 1 : 160. That these are R antibodies is shown by the low S agglutinin content of the R serum (2 units in the 1 : 5 dilution). Suspension II gives a similar curve with S serum (Fig. 7), but with a single broad zone whose optimum coincides with RS equivalence at 1 : 80. Compared with Suspension I, the reduction of S agglutinin in concentrated sera is less effective, and that of RS agglutinins slightly more effective. The rapid agglutination of the suspension by the R serum suggests a comparatively high S agglutinin content of the R serum, and the more effective absorption of the RS agglutinins suggests that the exposure of R antigen on the surface of Suspension II is relatively great. The optimum of the R curve at 1 : 10 corresponds to the S equivalence point. But since the same serum at 1 : 10 contained only 1 unit for Suspension I, the 12 units absorbed from this dilution were presumably R antibodies.

The behaviour of a salt- and heat-sensitive SR *abortus* variant is compared

with that of a fully smooth strain in Figs. 8 and 9. In 8, the SR gives a sharp optimum with S serum, equivalent for SR agglutinins, but incapable of removing more than a few S agglutinins even from low serum concentrations. SR agglutination with R sera is marked ; there is no definite optimum, but the two limits of the broad zone (1 : 5–1 : 40) are marked by the S and the SR equivalence points.

Variation in the absorbing power of a given antigenic surface.—In 9, the optimum of the S form with the S serum corresponds to the S equivalence point, and the suspension is capable of absorbing about one SR unit from the S serum. It can remove 5 SR units at the equivalence point of the R serum, and 18 of 22 units in R serum 4 times as concentrated. Its agglutination by R serum appears to be achieving an optimum in neat serum, above the equivalence point of the scanty S agglutinins. The small amount of agglutinin in the rough serum for the S suspension suggests that the agglutinin for SR suspension is R. Similarly the fact that the fully S suspension cannot touch the agglutinins for SR in the smooth serum suggests that they also are R agglutinins. In this event, the different powers of absorption of R agglutinins by the same suspension is striking. The non-agglutinability of the S by R serum, in spite of the effective R groups in the S cell, suggests that they are overshadowed by S groups, which keep the suspension highly hydrophile, though the R antigens are sensitized by R antibodies. The feeble absorption of the R antibodies in the S serum may be due to some form of steric interference ; in the high concentration of S antibodies, the S groups may be covered with antibodies which, in a large measure, prevent the access of R agglutinins to the scanty R receptors on the cell surface. In this connection it must be mentioned that some anti-S and anti-R sera were found to agglutinate SR or RS suspensions very slowly or not at all. Yet the absorption of 40–70 per cent. of the S agglutinins from such sera, by a small dose of S suspension, left a strongly SR agglutinating serum. Titres of 1 : 50 appeared in apparently inactive sera and the optimal particulation time was decreased from 12 to 2 hours in others. This phenomenon is still under investigation, but the explanation appears to be limited to two hypotheses. Either the S antibodies in the unabsorbed antisera are in excess for the S-groups on the SR cells, and render them highly hydrophile, and the absorption of S antibodies interferes in part with the absorption of R antibodies ; or the phenomenon is referable to SSS-antibody complexes in the absorbed serum. Smooth Brucella suspensions readily lose soluble antigens in the suspending fluid, and it is possible that, even with washed suspensions, some SSS is liberated during the incubation of absorption mixtures. This SSS, being in excess of the low concentrations of antibody employed, forms soluble complexes with antibody. It can be shown (see Miles and Pirie) that the agglutination titres of a serum are raised if it contains homologous soluble antigen-antibody complexes, and that partially rough suspensions are susceptible to the action of these complexes. As far as could be determined in the experiments recorded in Figs. 6–11, there was no such unmasking of SR agglutination, nor was free SSS detectable initially in the S suspensions used. Nevertheless, the possibility of such an occurrence must throw some doubt on the quantitative measures of absorption in agglutination systems of this kind.

Effect of the R antigen in the agglutination of smooth bacteria.

The broad zone of agglutination in a smooth Brucella system is the resultant of at least two sensitization maxima. One is referable to the smooth antigens, the other to the rough. The R agglutinin response in the rabbit to markedly rough bacteria is relatively late, and later still to S bacteria. For example, the RS agglutinin titre of sera 1 and 2 in Figs 3 was $<1:10$. If an early S serum is used, containing few R antibodies, or if the exposure of R antigen in the S suspension is small, the agglutination zone is sharp (*e.g.* Fig. 8). Broader zones occur with greater exposure of R antigen (Figs. 6 and 7); the shape of the zones and the position of the equivalence point of S antisera agree with the hypothetical indications that, when the R antibody concentration is low, increasing exposure of the R antigen, as the S—→SR variation progresses, shifts the point of maximal R sensitization into more concentrated serum. Upper optima of the type shown in Fig. 4 are probably R optima, and the splitting of one zone, as in Fig. 5, is due to the reduction of R antibodies so that the point of maximal R sensitization was shifted decisively into more concentrated serum.

THE MONOSPECIFIC SERUM.

The existence of A and M antigen was inferred from the demonstration of monospecific agglutinins in the supernatant fluids of partially absorbed sera. Hitherto A and M have been considered as separate antigens, giving rise to antibody molecules exclusively determined by a and μ receptors respectively. Alternatively they may be determinates of an antigenic complex large enough to present to the *in vitro* action of antibody relatively discrete areas of A and M, but with patches of A and M occurring together in areas comparable with that covered by an antibody globulin molecule, *i. e.* the surface is (AM), not (A + M). Then, adopting Beinl and Haurowitz's (1930) hypothesis of antibody formation, a globulin molecule built up in the region of the minor antigenic determinant on the surface of the antigen complex should contain both a and μ receptors, *i. e.* ($a\mu$), with receptors for the major determinant predominating.

The monospecific *abortus* serum, *e. g.*, fails to agglutinate *melitensis* because the A groups are so scanty. Either the area made hydrophobe by their sensitization is not sufficient to overcome the hydrophile tendencies of the rest of the bacterial surface (Eagle's hypothesis), or the links made by antibody between approximated A areas on adjacent bacteria are too few to hold the bacteria together against the dispersive forces at work in the suspension (Marrack's hypothesis). On the other hand, if only ($a\mu$) antibodies, predominantly a in type, were present, they would be attached firmly to the few A areas, and feebly to a considerable area of M antigen. The amount absorbed would be large, though presumably the antibody on the M areas would not be altered enough to become hydrophobe (Eagle's hypothesis), or, the scanty μ receptors would not form links strong enough to bind bacterium to bacterium (Marrack's hypothesis). But, as Table I shows, the absorption of monospecific sera by heterologous suspensions is very small. The antibody is therefore functionally a and not ($a\mu$) in type. However, the fact that the monospecific antibody is a alone does not preclude the possibility that *Br. abortus* may be represented by (AM) rather than (A + M), for, as Morgan (1936) suggested,

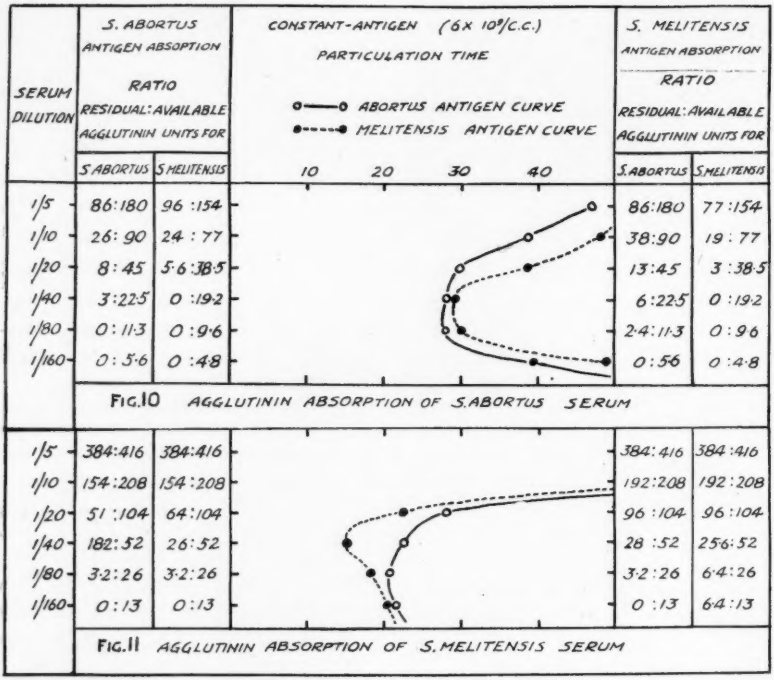

SERUM DILUTION
S. ABORTUS ANTIGEN ABSOPTION RATIO RESIDUAL: AVAILABLE AGGLUTININ UNITS FOR
S. ABORTUS
S. MELITENSIS
CONSTANT-ANTIGEN (6X 10⁹/C.C.) PARTICULATION TIME
ABORTUS ANTIGEN CURVE
MELITENSIS ANTIGEN CURVE
10 20 30 40
S. MELITENSIS ANTIGEN ABSORPTION RATIO RESIDUAL: AVAILABLE AGGLUTININ UNITS FOR
1/5 86:180 96:154 86:180 77:154
1/10 26:90 24:77 38:90 19:77
1/20 8:45 5·6:38·5 13:45 3:38·5
1/40 3:22·5 0:19·2 6:22·5 0:19·2
1/80 0:11·3 0:9·6 2·4:11·3 0:9·6
1/160 0:5·6 0:4·8 0:5·6 0:4·8

FIG. 10 AGGLUTININ ABSORPTION OF S. ABORTUS SERUM

FIG. 11 AGGLUTININ ABSORPTION OF S. MELITENSIS SERUM

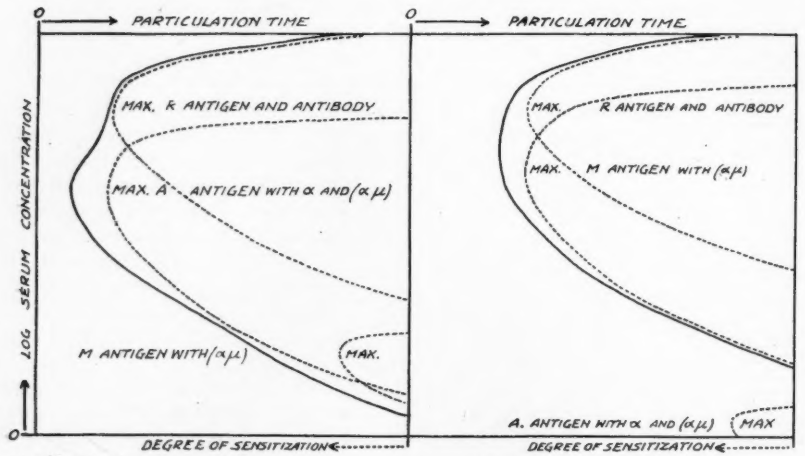


FIG 12 (a) HOMOLOGOUS SR SYSTEM (b) HETEROLOGOUS SR SYSTEM

FIGS. 10 and 11.—Constant-antigen particulation times of S *abortus* and *melitensis* suspensions with homologous and heterologous sera. Serum dilutions recorded on a logarithmic scale.

FIG. 12.—Hypothetical sensitization maxima and the resultant agglutination optima of (*a*) SR *abortus* and (*b*) SR *melitensis* suspensions with anti-*abortus* serum.

the early antibody response to an antigen of the type AM would be a, *i. e.* antibody to the major determinant only. Later, when the antibodies were richer in receptors, both a and μ would be present in the same particle. These ($a\mu$) antibodies, by reason of their power of combining with both A and M antigens (*cf.* Meyer, 1936) and the comparative wealth of receptors, would be more avid than the a antibody. Absorption of a mixture of ($a\mu$) and a with a small dose of *melitensis* would leave the less avid a behind as a monospecific antibody solution.

The conception of the smooth sensitization maxima in positions determined by the relative values of $[a]/[A]$ and $[\mu]/[M]$ does not apply to all the absorption phenomena of the Brucella systems. Graded cross absorption of smooth anti-*abortus* (Fig. 10) produced a monspecific *abortus* serum in dilutions of 1 : 40 and 1 : 80 (6 and 2·4 units), and in 1 : 20, with 13 *abortus* and 3 *melitensis* units, a mixture that would be monospecific on 4-fold dilution. Now if the A groups of *melitensis* are insufficient for its agglutination, the cross agglutination of *melitensis* by unabsorbed *abortus* sera, to titres nearly as high as the homologous agglutination, on the ($a + \mu$) hypothesis, must be due to high level of μ antibodies in the *abortus* serum. That is, $[a]/[\mu]$ is in the region of unity. An absorption with a *melitensis* suspension in which $[A]/[M] = 1/20$ should leave a large amount of a, even assuming that the A groupings are absorbing disproportionately large amounts from the relative excess of a antibody. Moreover, the absorption of such a serum by a suitable dose of *abortus* where $[A]/[M] = 20/1$, should leave a strong solution of μ monospecific for *melitensis*, whereas (Fig. 10) in fact a weak solution of a (3 units) is left in the 1 : 40 dilution.

Again, in Fig. 11, the *melitensis* serum had equal initial titres for *melitensis* and *abortus*, and absorption revealed no clear cut monospecificity, though μ predominates slightly in the 1 : 80 homologous absorption (6·4 units as against 3·2). In general it was found that early bleedings of good titre yielded monospecific sera on heterologous absorption, but bleedings after prolonged immunization were unpredictable in their behaviour. In this connection it is noteworthy that the highly differentiated monospecific sera recorded by Veazie and Meyer (1936) were prepared from antisera made by a single course of injections. The avidity of the antibodies, and therefore presumably the number of receptors per globulin molecule, increases with prolongation of immunization. Taking *abortus* sera as an example, at first *abortus* and *melitensis* agglutinins appear simultaneously, and, though titres reach their maximum after a few injections, the optimal agglutination velocity continues to rise to treble or quadruple its initial value. After a sufficient number of courses, in 50 per cent. of the rabbits there appear precipitins for homologous SSS, and later for heterologous SSS ; and in constant-antibody titrations of standard solutions of SSS, both the optimal precipitating velocity and the optimally reacting concentrations of SSS increase with further immunization. By postulating that the serum globulin molecules are modified by functional receptors in the following order as immunization proceeds : a agglutinin, ($a\mu$) agglutinins, a precipitins, ($a\mu$) precipitins, each more avid than the preceding, the agglutination and precipitation phenomena that do not fit the simpler ($a + \mu$) conception of antisera are readily explained.

The smooth *abortus* sensitization maximum on this hypothesis is due to the action of ($a\mu$) and a antibodies, the A antigen being mainly effective in the production of agglutination. Homologous partial absorption will leave a little of the less avid a. Heterologous partial absorption will leave more of the a, since the effective A antigen is small (*cf.* Fig. 10). In riper sera (the *melitensis* serum of Fig. 11 was obtained after five courses of immunization, and contained precipitins) the antibodies with a single type of receptor are usually scanty, and monospecific sera cannot be readily produced by bacterial absorption.

Hypothetical considerations.—The non-agglutination of heterologous suspensions by monospecific sera can be readily explained on Eagle's hypothesis ; the minor antigenic surface being only 5 per cent. of the total, its sensitization is presumably insufficient to decrease the salt-stability of the suspension beyond the critical point. However, there is strong evidence from the study of other systems in favour of the lattice structure of an agglutinated mass (Marrack, 1938 ; Duncan, 1938). On this basis it is suggested that in Brucella monospecific systems the linkages between minor antigenic groups on the heterologous bacteria are insufficient to hold the individual bacteria in clumps. Marrack (1938, p. 169) has calculated that agglutination of flagellated typhoid bacilli can occur when the antibody present could at most cover only 2 per cent. of the somatic surface of the bacilli, let alone the relatively much greater flagellar surface, and it is arguable that the 5 per cent. minor antigenic surface should be enough for the agglutination of heterologous Brucella organisms. The comparison is not necessarily valid, for Marrack's figure of 2 per cent. represents a proportion of the *total* surface ; the 5 per cent. Brucella figure, derived from absorption tests, represents the fraction of the *antigenic* surface, which may itself cover only a fraction of the total surface. Moreover, it is possible that the patches of minor antigen are so irregularly disposed that when two bacteria are approximated, only a few of the minor antigen patches present on the one are near enough to similar patches of the other to allow linkage by an antibody molecule. Thus, though absorption might take place over 1/20 of the antigenic area, linkage would be possible with a much smaller fraction, most of the groups on adjacent bacteria being separated by distances greater than the length of an antibody molecule. There is thus nothing inherently improbable in the "lattice" interpretation of Brucella monospecific sera, though more exact definition of the antigens may necessitate its quantitative modification.

ANALYSIS OF THE BRUCELLA CONSTANT-ANTIGEN AGGLUTINATION ZONE.

In the light of the above findings, the agglutination phenomena of smooth or apparently smooth suspensions by their respective antisera may be described in terms of A, M and R antigens, and a, μ, ($a\mu$) and R antibodies. The hypothetical constant-antigen titration of *abortus* and *melitensis* against an anti-*abortus* serum is represented in Figs. 12, *a* and *b*. The dotted lines represent the degree of sensitization of the different antigenic components, and the continuous line the standard particulation time of the suspensions in varying serum concentrations. In concentrations effectively sensitizing one antigen

only, the particulation velocity parallels the degree of sensitization ; where two antigens are simultaneously sensitized, the particulation velocity is dependent on the combined effects. There is a small exposure of R antigen on the organism. The serum contains a moderate amount of R agglutinin and the ($a\mu$) content is high, so that the R maxima, and the major antigen maxima in each titration, are similar, though the heterologous optimum occurs later and in higher serum concentrations (*cf.* Fig. 10). The minor antigenic maxima, since they are ineffective when acting alone with heterologous monospecific sera, can be assigned nothing more than auxiliary parts in direct agglutination. The M sensitization maximum in *abortus* slightly enhances the agglutination due to the main A antigen in the lower regions of the curve ; but in the analogous A sensitization of the *melitensis* the a antibody is in such comparative excess that the maximum is outside the range in which its auxiliary effect might be manifested.

SUMMARY AND CONCLUSIONS.

The foregoing analysis of smooth Brucella agglutination has been made to test the hypothesis that the surface of *Br. melitensis* and *Br. abortus* consist of M and A antigens. The agglutinin response of the rabbit is predictable only in terms of whole bacteria, *i. e.* mixtures of A and M, and is, moreover, highly variable. The existence of the corresponding antibodies, a and μ, can be inferred only from the behaviour of the hypothetical antigen mixture. The difficulty of unambiguously describing two unknowns in terms of two other unknowns is to some extent overcome by studying the particulation time curves of the various systems as a whole. The applicability of a particular empirical solution can be gauged more effectively from these than from the determinations of optimal reacting proportions, maximum absorbing doses, or (Heidelberger and Kabat, 1934) the maximum nitrogen absorbable from antisera ; methods which, unless there is already a precise definition of one reagent in the system, can reveal only those quantitative differences that are based on gross qualitative differences. Nevertheless, though the picture presented is consistent, it cannot be substantiated until the A and M antigens are isolated as separate substances, free from contamination with one another.

The absorption of monospecific sera, both by homologous and heterologous suspensions, offers a direct means of measuring the ratio of A to M on the antigenic surface of Brucella strains, and the method described should facilitate the antigenic definition of the various serological subspecies in the Brucella group. The surface of the smooth strains of *abortus* and *melitensis* studied consists at least of A and M antigen ; the minor antigen is present in proportions varying from 1 : 40 to 1 : 10. The lower ratio occur in *Br. abortus*. Pending confirmation of this fact, the A : M ratios in *abortus* and *melitensis* are assumed to be about 20 : 1 and 1 : 20 respectively.

Smooth strains may undergo slight S—→SR variation with a consequent exposure of R antigen on the surface that is insufficient to render the organism agglutinable by heat or saline or to lower the anti-smooth titre, but which may be detected by a slight increase in anti-R titre and a drop in the content of specific soluble substance. The agglutination zone of such strains is the

resultant of reaction of A, M and R antigen with their respective antibodies. Doubling of the zone occurs when the exposure of R antigen and the relative proportion of R agglutinins are favourable ; the R optimum occurs in low, the (A + M) optimum in high serum concentrations.

In certain tests the R antigens exhibited different agglutinability and absorption powers according to the amount of S antigen and smooth antibodies present in the system, and though these effects were not observed with most of the sera tested, their occurrence emphasizes the need for caution in the interpretation of quantitative absorption tests.

The probable response of the rabbit to this mixture of A, M and R antigens is, firstly, the formation of antibodies with receptors to the major smooth antigen, either a or μ ; secondly, antibodies with both receptors, ($a\mu$), and antibodies to the R antigen. Absorption by small doses of heterologous organisms remove the ($a\mu$), leaving monospecific sera containing a or μ alone.

The monospecific Brucella antiserum is readily interpretable in the light either of Eagle's or of Marrack's hypothesis of specific antigen-antibody flocculation.

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THE PROPERTIES OF ANTIGENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*: I. CHEMICAL AND PHYSICAL PROPERTIES OF BACTERIAL FRACTIONS.

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ALTHOUGH the immunological characters of the *Brucella* group of micro-organisms have been studied in some detail, comparatively little thorough work has been done on the isolation and chemical characterization of the antigens. Favilli and Biancalani (1932, 1934) studied the alcohol precipitable material in that part of a hot saline extract of agar-grown bacteria which was not precipitable by trichloracetic acid or by uranyl acetate. This material contained nitrogen, and gave reducing sugar and an osazone on hydrolysis. It had a low precipitin titre and failed to immunize rabbits. Huston, Huddleson and Hershey (1934) described the isolation from dried agar-grown *Br. melitensis* of a specifically precipitable substance (their S substance) that was free from both protein and polysaccharide. They also isolated inactive proteins, polysaccharides and lipoids from the three main *Brucella* species. Hershey, Huddleson and Pennell (1935) reported the isolation from *Br. abortus* of a substance resembling the acid hydrolysed S substance of *Br. melitensis* which gave specific precipitates at 1 : 2,000,000 with antisera. It was optically inactive and they described it as containing $\pm$ 2 per cent. of nitrogen. Pennell and Huddleson (1937) have described in detail the properties of a material which they call the endo-antigen. This is prepared from ground dried organisms by incubation with trypsin, and after removal of the material precipitable by trichloracetic acid, precipitation with alcohol. The extreme variability in the properties of this substance led to the conclusion that it is by no means pure, and reference to it will be postponed until we have described the chemical properties of our own products.

Topping (1934) described a few chemical and serological properties of a feebly antigenic " nucleoprotein " material that was precipitated by acid from a dilute alkaline extract of dried *Br. abortus*, and of a " polysaccharide " material that precipitated with alcohol from the acid supernatant ; this had a

precipitin titre of 1 : 2000. Schapira (1936) has studied a similar product purified by precipitation with phosphotungstic acid, and Reiter (1936) from all three species has prepared feebly antigenic substances of end-titre 1 : 50,000, by a method similar to that of Topping. Higginbotham and Heathman (1936) extracted precipitating substances of end-point 1 : 10,000 from the three species; some of these appeared to be serologically distinct by precipitin tests. Detailed animal tests have been carried out by Gwatkin (1935) with the material precipitated by alcohol from the filtered extract of *Br. abortus*. Lisbonne and Monnier (1936) and, more recently, Pop, Damboviceanu, Barber and Marinov (1938) have prepared toxic and antigenic solutions, similar to the " complete antigens " of Boivin and Mesrobeanu (1935), by dialysing the trichloracetic acid extracts from the well-washed organisms of all three species. The technique of purification used by all these workers is relatively simple, and experience suggests that a mixture of large molecules would not necessarily be fractionated by these procedures. This expectation is borne out by the low serum precipitation titres reported and by the variability of the products described. Our attempts to isolate the antigens from bacteria of the *Brucella* group started in 1932 and have proceeded intermittently since then. Recently we have developed methods by which material of a relatively constant chemical composition and serological behaviour may invariably be isolated from cultures of smooth *Br. melitensis*. Only *Br. melitensis* has been studied in detail. Some fractions of *Br. abortus* were prepared for serological comparison, but the more exact fractionation of *Br. abortus* will be reported in a later paper.

It was our original intention to isolate the A and M antigens postulated by Wilson and Miles in 1932. This we have not yet accomplished; the present paper describes the " native antigen " of *Br. melitensis* and derivatives of it. There is, of course, no conclusive evidence that the " native antigen " is not an artefact, nor is there evidence that it and the substances derived from it are pure; but since there is apparently no technique whereby such evidence could be obtained, it seems justifiable to publish these results in their present form. It will be obvious that this work is in part an amplification of the papers already quoted which have appeared while the research was in progress, and that these papers have been of great use to us, although we may often disagree with their conclusions.

PREPARATION OF BACTERIAL SUSPENSIONS.

Br. melitensis, strain Younovitch 6 (Y6) and *Br. abortus*, strain Kristensen 25 (K25) were studied. Subcultures for each harvest were tested for agglutinability by salt, heat and smooth antiserum (Pandit and Wilson, 1933). Early in the work certain subcultures were found smooth by these tests and yet yielded very little SSS. The symbol SSS denotes any specifically precipitable soluble material obtained from the organisms. A routine test for extractable SSS was made as follows: Two-day liver agar slopes were washed off in 3 c.c. water, the bacterial concentration in 1 c.c. of wash-off determined by an opacity method, and the remainder extracted for 1 hour at 60° C. Extracts from satisfactory subcultures had a constant-antiserum precipitin titre of about 1 : 10 for every 10^9 bacteria per c.c. Two unsuccessful attempts were

made to select a strain of Y6 with a high SSS yield. Subcultures of 20 colonies from a plate culture were tested as described above. The distribution of yields from unit bacterial suspension was approximately normal, with a mean of 1 : 7·5. The scatter of values round the mean was small, with a total range of 1 : 5·5 to 1 : 9. Subcultures of strains of low, mean and of high yields were again tested, 20 colonies from each, but the resulting distributions did not differ significantly from the original distribution.

The rate of S→SR→R variation of Y6 and K25 is greater in broth than on solid media ; after 4–5 days 50–70 per cent. of the organisms may be SR. The SSS-poor SR variants were avoided by harvesting the growths from liver extract agar (Huddleson *et al.*, 1927), which can be depended on for good yields if heavy inocula are used. A 2-day liver agar slope in 5 c.c. broth was flooded over 150 c.c. agar in " medicine flats " of 40 oz. capacity, and incubated for 4–5 days—one day horizontal, and 3–4 days tilted to leave 90 per cent. of the surface free from fluid. Both longer incubation and the use of more concentrated liver extract gave better growth, but no bigger total yield of SSS. In one comparative test the 26 g. bacteria from double strength medium gave 1·2 g. crude SSS, whereas the 17·6 g. from the same number of bottles of single strength yielded 2·2 g. SSS. The SSS yield of Y6 was best in air ; it fell off with the following gas-mixture series ; air > 38 per cent. O_2 + 62 per cent. N_2>8 per cent. CO_2 + 92 per cent. air>limited air supply. The yield from K25 was optimal in 5–10 per cent. CO_2.

The apparent macroscopic purity of cultures was confirmed by examination of stained smears from each bottle, the pure growths soaked in distilled water for 1–2 hours, washed off and filtered through glass wool to remove flakes of agar gel. The amount of SSS left in the agar medium after the wash-off was too small to be worth recovering by extraction.

One of the most difficult technical features of the isolation of SSS from bacteria grown on liver agar was the removal of agar derivatives. A few batches were grown on inspissated serum, lemco gelatine and in liver broth for comparison. On broth the yield was very low ; when 5 litres of liver extract were made into broth the yield was about 2 g. dry bacteria, whereas when made into liver agar the yield was about 10 g. On inspissated serum, made with 75 per cent. ox serum and 25 per cent. double strength liver extract, the yield was moderately good, though the wash-offs were rich in ox-serum proteins. As the work proceeded, however, it became possible to remove agar from preparations with certainty, and it was therefore more convenient always to grow the bacteria on agar media.

KILLING AND EXTRACTION OF BACTERIAL SUSPENSIONS.

The water-soluble and indiffusible part of the bacterial mass of *Br. melitensis* grown on agar and killed with chloroform, phenol or acetic acid contains a large number of different substances. Part of this mixture is simply agar which has undergone some disintegration ; the agar is largely in two forms, both of which are precipitated by alcohol in the presence of traces of salts. One form is precipitated at 1/10 of saturation by ammonium sulphate, whereas

the other is analogous to " kanten " (Pirie, 1936) and precipitates at about half saturation. Each fraction can be obtained free from the SSS, and each will give the colour reactions characteristic of agar (Pirie, 1936) to the same extent as agar. Further evidence that these substances are derived from agar and are not bacterial products comes from the fact that similar materials are not present in batches of bacteria grown on broth, inspissated serum or gelatine.

The bacterial products fall into four easily distinguishable fractions, each of which may be contaminated with agar, but can always, although not always very easily, be freed from it by repeated fractionation. These fractions are : the SSS, a mixture of lipoids, nucleic acid and nucleoprotein. The nucleoprotein preparations usually carry serological activity, but there is no evidence that this is not always due to contamination with SSS, for the latter can be recovered from an enzyme digest of active nucleoprotein ; there is likewise no evidence that only one nucleoprotein is present. These nucleoproteins have been prepared by methods involving the use of alkalis and of alcoholic precipitation, which might well have robbed them of antigenicity. Some attempts have been made to prepare unmodified nucleoprotein, but we will not present here any evidence bearing on the antigenicity of *Brucella* nucleoprotein in the native state.

The specific soluble substance (SSS) can exist in three clearly definable states of complexity with similar but distinguishable chemical, physical and serological properties. It is convenient to use different methods of isolation when the less complex forms of SSS are required, and for this reason the methods will be described separately. As in all work involving the separation of components from a mixture of large molecules, round-about methods of procedure are necessary, for it is seldom found that the concentration of salt or solvent which is necessary to precipitate a pure substance from solution will be the same as that which precipitates it from a mixture.

Batches of Y6 differ chemically from one another for reasons that we do not at present understand. There is variation not only in the constituents of the bacterial cell, but also in the extent to which soluble derivatives of the medium appear in the fluid washed off its surface. For this reason it is necessary to describe the principles that are used as a guide in the isolation of SSS rather than methods which should be adhered to rigidly.

PREPARATION OF NATIVE ANTIGEN.

The SSS, in its least modified form, is a complex of a phospholipid mixture (PL1) with a substance giving the Sakaguchi reaction for arginine (S) and a substance which may, for the time being, be described as a highly aggregated formyl derivative of an amino-polyhydroxy compound (AP). The whole complex is referred to below as " native antigen " or by the symbol [PLAPS].*

* These letters are intended simply as symbols and, although the choice of letters is, to some extent, governed by the chemical properties of the substance referred to, it is not to be supposed that the letters summarize accurately what is known about the constitution of these substances. These symbols have no connection with the symbols used by other workers in the same field.

Extracts which contain this [PLAPS] are opalescent, viscous, and show anisotropy of flow when fresh, but a number of agents, such as drying, freezing, exposure to organic solvents and to acids or alkalis, disintegrate the complex giving the free lipoid and AP in various stages of disaggregation.

For the isolation of SSS in its least modified form 2 per cent. phenol is the most suitable killing agent. The suspension of bacteria with phenol was left at 0° C. for some days or weeks, and centrifuged at 3500 r.p.m.; the bacterial mass was extracted again with about half the original volume of water. From the residue disaggregated AP can be isolated by the acetic acid extraction technique, which will be described later.

To each litre of extract 430 g. of ammonium sulphate was added; a bulky precipitate separates immediately. If the mixture is now centrifuged part of this sinks and part floats. This difficulty may be overcome by filtering or, more conveniently, by carrying out the precipitation in a stout bottle and removing the bubbles attached to the precipitate with a filter-pump. The fluid after this precipitation contains agar, lipoids and nucleic acid, but no SSS. The precipitate was dissolved in 20 or 30 times its volume of water, and half the final volume of saturated ammonium sulphate solution was added. Although the purified SSS is not precipitated by 1/3 saturated ammonium sulphate, almost all the SSS comes out with the precipitate at this stage. The fluid should, however, be kept for subsequent recovery of a little more AP. The precipitate was again dissolved in water, re-precipitated by the addition of 1/3 of a volume of ammonium sulphate solution and the process repeated. This precipitate is mainly agar and protein, but as much as 1/5 of it may be [PLAPS]. It is possible to separate this mixture by further precipitations with ammonium sulphate if solutions containing only 2–3 mg. of indiffusible material per c.c. are used. The procedure is tedious, and it is generally more profitable to use the material for the preparation of disaggregated AP.

To the 1/4 saturated ammonium sulphate solution enough ammonium sulphate was added to raise the concentration to 40 per cent. of saturation. The precipitate was collected and dissolved in water. It is possible to separate this material into a number of fractions by adjustment of the pH at various values between 3 and 6, or by precipitation with concentrations of ammonium sulphate between 30 and 40 per cent. of saturation. These fractions differ considerably in physical and chemical, but not consistently in serological properties. No appreciable purification has so far been effected by these procedures, and the characteristic properties of the precipitates are those of the impurities rather than of SSS.

Centrifugal fractionation is the only method that has been found satisfactory for the isolation of the intact antigen from this mixture. The 40 per cent. ammonium sulphate precipitate was dissolved in sufficient water to give a neutral solution containing 0·5–1·0 per cent. of indiffusible material, and this was centrifuged at 14,000 r.p.m. for half an hour in a rotor 15 cm. in diameter. If a more concentrated solution is used, sedimentation of the same proportion of SSS (about 90 per cent.) is slower. The supernatant fluid contains a little antigen, which may be recovered, and variable amounts of the inactive bacterial constituents. The turbid centrifuge pellets were dissolved in water to give a 0·5 per cent. solution and centrifuged again in the same way; the process

was repeated after removing some material by centrifuging the 0·5 per cent. solution for 1–2 hours at 3500 r.p.m. On the third high-speed centrifugation it is found that the supernatant contains little except a trace of ammonium sulphate. The pellets are now yellowish and clear, or at most slightly turbid. If examined in polarized light they are found to be birefringent at first, but this disappears on standing. The birefringence can be restored by re-orientating the particles by stirring or tapping. It is material prepared in this way that will be referred to as " native SSS " or [PLAPS].

PREPARATION OF DISAGGREGATED ANTIGEN.

When only the disaggregated antigen is required bacteria may be killed in a number of different ways, and no substantial differences have been found between preparations made from cultures killed by heating or by the action of chloroform, phenol, or acetic acid. Shaking with $CHCl_3$ not only killed the bacteria, but brought the bulk of the SSS into solution ; 5 per cent. acetic acid had the same effect ; 5 to 20 per cent. trichloracetic acid, 75 per cent. alcohol, water at 100° C., water at 56° C. and saturated urea solution were less good in leaching the SSS, in that order. Caprylic alcohol was an effective killing agent, but reduced the suspensions to a thick slime, only " cleaned " by the addition of acetic acid. Precipitation of the wash-off with 50 per cent. acetone, extracting at 20° C. for 1 week with pure acetone and drying with ether yielded a powder that was readily dissolved by trypsin (*cf.* Raistrick and Topley, 1934), but the larger antigenic complexes were partly destroyed by this method. As a matter of convenience we now add to the bacterial suspension some chloroform and enough acetic acid to raise the concentration to 25 per cent., and then leave the mixture for some days. By this technique much bacterial protein is left unextracted. The SSS and the agar breakdown products are distributed between the bacterial residue and the fluid but, although the ratio of agar to SSS is greater in the fluid than in the residue, the difference is not sufficiently great to make it worth while to work up the two fractions separately.

The mixture was centrifuged till clear ; generally this takes two hours at 3500 r.p.m. Acetic acid was now added in small quantities to the bacterial residue, with thorough stirring till the acetic acid concentration had been raised to about 80 per cent. The mixture was now diluted with 80 per cent. acetic acid till it is a thin cream. For 30 g. dry weight of bacteria about a litre of fluid is convenient. It was centrifuged (and generally centrifuges easily), giving a clear fluid containing the AP. If the mixture does not centrifuge easily one volume of alcohol is added and the mixture is centrifuged again ; under these conditions the centrifugation is invariably easy. The supernatant, if alcohol has been added, contains no SSS but much lipoid is extracted. This step is essential with suspensions from partly rough cultures. After either preliminary treatment the solid was re-suspended in 80 per cent. acetic acid and centrifuged, and the extraction was repeated by suspending the bacteria in enough water to make a thin cream, adding four times that volume of acetic acid and centrifuging. The 80 per cent. acetic acid solutions were mixed with 1 or 1·3 volumes of alcohol. There is a heavy precipitate of

crude SSS and agar. This was centrifuged off, dissolved in water, and added to the original 25 per cent. acetic acid extract of the bacteria. The bacterial residue still contains some SSS which may be extracted (together with large amounts of protein) at neutrality, and still more may be recovered from the residue by incubation with trypsin. If the earlier extractions with acetic acid have been thorough the residual quantities hardly repay the labour of extraction.

The extract was now precipitated with ammonium sulphate, using the technique used for the isolation of [PLAPS]. The precipitate was dissolved and re-precipitated at 1/3 of saturation with ammonium sulphate. From the mother liquor a small amount of antigen may be recovered by raising the ammonium sulphate concentration to 40 per cent. of saturation. This was added to the first precipitate. By carrying out the precipitation in two stages in this way it is possible to leave a much larger proportion of the agar-breakdown products in the fluid than if the precipitation is carried out at 40 per cent. of saturation in one step.

A number of alternative procedures for the preliminary fractionation have been tried and found unsatisfactory. Precipitation with alcohol at various pH values leads to no fractionation, for all the materials except the lipoids are readily precipitated. The removal of inert material by precipitation at pH 3 may be useful, but the precipitate, which consists largely of protein and lipoid, often carries out with it a large amount of SSS. This may be recovered by incubation with trypsin and subsequent treatment with glacial acetic acid, but nothing is gained by isolating a small amount of SSS by this roundabout method.

Ammonium sulphate at 40 per cent. of saturation will precipitate from the bacterial extract between 10 and 20 per cent. of the initial dry weight of the bacteria. This precipitate may contain as much as 50 per cent. of agar breakdown products besides protein and lipoid. Part of the SSS may be separated from the mixture by the use of acid, alcohol and ammonium sulphate directly, but the yield is greater if most of the protein is first removed by incubation with trypsin. The ammonium sulphate precipitate, suspended in as little water as possible, was dialyzed to remove most of the salt. The turbid solution was diluted till it contained 1–2 per cent. of indiffusible components, and incubated at pH 8 and 37° C. with about 0·3 per cent. " Pancreatin " (Merk). The incubation was continued for 36–48 hours, with frequent readjustment of pH during the first few hours. The SSS and agar were now precipitated twice over by the addition of three volumes of alcohol ; some alcohol-soluble material is carried down, but cannot be removed at this stage by further alcohol precipitations. The precipitate was dissolved in a small amount of water and four volumes of acetic acid added ; if a precipitate separated it was centrifuged off and kept, as more antigen may be isolated from it. The fluid was mixed with 1–1·3 volumes of alcohol, centrifuged, and the solid was redissolved in water and re-precipitated with alcohol and acetic acid. This time, to make the precipitate flocculate, it may be necessary to add some salt (a few drops of a 0·5 per cent. alcoholic solution of calcium chloride will generally suffice). The solid was now dissolved in 20–30 times its volume of water. On the addition of half this volume of a saturated solution of ammonium sulphate, a precipitate

which may consist of almost pure agar separated ; this precipitate should be retained for it frequently contains some antigen. To the fluid 1/4 volume of ammonium sulphate solution was added ; the antigen precipitated and more agar was left in the supernatant. The precipitate was now dissolved in water and exactly 0·6 volumes of saturated ammonium sulphate solution was added. The precipitation of this purified SSS takes place immediately, and the critical concentration of ammonium sulphate is sharply defined at each temperature. At 15°–20° C. precipitation is complete with 0·6 volumes, whereas there is no precipitation with 0·55 volumes. The precipitated SSS redissolves when the suspension is cooled to 0° C. A few other large molecules are known to behave in a similar manner, *e. g.* methylated glycogen (Bell 1935), hæmoglobin (Green, 1931), and some plant viruses (Bawden and Pirie, 1938*b* ; Pirie, Smith, Spooner and McClement, 1938). The property is, however, relatively rare, and it is valuable for the removal of the last traces of agar, which precipitates with substantially the same concentration of ammonium sulphate at 0° C. and at 20° C. The mixture containing 0·6 volumes of ammonium sulphate was therefore cooled to about — 5° C. and centrifuged for half an hour at 3500 r.p.m. in a centrifuge kept at or below 0° C. The supernatant was poured off and allowed to warm up to room temperature ; as it warmed the SSS precipitated and could be centrifuged off. The SSS, when precipitated in this way, is an almost clear, pale yellow jelly ; this, if free from agar, will dissolve very easily in a small quantity of 1/3 saturated ammonium sulphate solution at room temperature. If it does not, the process of precipitation and cold centrifugation should be repeated.

No impurities have been recognized in material purified in this manner except a trace of protein, which is most easily removed by a method based on that of Sevag (1934), as modified by Heidelberger *et al.* (1936). The ammonium sulphate precipitate was dissolved in about 20 volumes of *M*/3 acetate buffer at about pH 4·6, and the solution was shaken mechanically with 1/5 volume of a 1 : 5 mixture of chloroform and butyl alcohol for about two hours. After centrifuging the clear aqueous supernatant was shaken again with fresh solvent mixture, and this process was repeated till only traces of emulsion separated at the interface on centrifuging. Some SSS could be recovered from the emulsion by shaking with fresh buffer solution. To the mixed supernatant fluids 0·6 volumes of saturated ammonium sulphate solution was added. It is prudent to cool the mixture to 0° C. to make sure that all the agar has been removed, for sometimes, after the removal of the protein, a trace of agar becomes removable by the technique used before. When warm the SSS was centrifuged off and dissolved in a little water ; it may be freed from ammonium sulphate either by dialysis, or by repeated precipitation with methyl alcohol. In the absence of salts neutral SSS is not precipitated by the addition of four volumes of methyl alcohol, but acid SSS is precipitated. For the second precipitation the solid was, therefore, dissolved in *N*/10 HCl. After three to four more precipitations the supernatant is free from both acid and ammonium sulphate. The clear yellowish pellet was dissolved in a little water and neutralized with dilute sodium hydroxide solution. The SSS has an apparent equivalent weight of about 8000. The neutral solution was then frozen and dried while frozen in a vacuum desiccator. Material prepared in this way will be referred

to as AP. The yield of AP has never been greater than 10 per cent. of the dry weight of the bacteria taken, and it is generally about 5 per cent.

RECOVERY OF ANTIGEN FROM RESIDUES.

At various stages in these preparations precipitates have been removed from solutions 1/4 or 1/3 saturated with ammonium sulphate and fluids have been discarded that were 2/5 saturated. From these more SSS can be recovered; it is possible to isolate the SSS in the aggregated state, but it is easier to disaggregate it before the isolation. Any active material in the fluids was precipitated by raising the ammonium sulphate concentration to 1/2 saturation and this precipitate was added to the other precipitates, and the mixture, dialysed for a few hours to remove most of the ammonium sulphate. The fluid generally contained some insoluble material, but without separation it was neutralized, boiled and incubated with trypsin at pH 8. Any material that was insoluble after two days was removed and the antigen was precipitated by the addition of 3–4 volumes of alcohol; this precipitate was dissolved in four or five times its bulk of water and five volumes of acetic acid was added to cause disaggregation. If a precipitate separated it was removed, and the SSS was precipitated by adding to the fluid its own volume of alcohol. The precipitate was centrifuged off, dissolved in water and acetic acid as before and reprecipitated with 1·3 volumes of alcohol.

The preparation at this stage will contain little except SSS, agar and nucleic acid; this mixture can be fractionated by ammonium sulphate precipitation following exactly the technique already used. Ammonium sulphate should not be added to fluids containing more than 0·3 per cent. of solid or the separation will not be sharp, and the fluids should be kept just alkaline with ammonia or some of the nucleic acid will precipitate with the SSS.

CHEMICAL AND PHYSICAL PROPERTIES OF THE SSS IN ITS LEAST MODIFIED STATE.

Solutions of the SSS when isolated by mild procedures, *i. e.* precipitation with ammonium sulphate or sedimentation on the high-speed centrifuge, are opalescent and show anistropy of flow. In the latter respect they resemble some plant viruses closely (Bawden and Pirie, 1937, 1938*a*, *b*). The anisotropy of flow can easily be observed when a fresh 0·1 or 0·2 per cent. solution in a tube 0·5 to 1 cm. wide is agitated gently between crossed nicol prisms. In this respect, therefore, it resembles the viruses, and the phenomenon can be more easily demonstrated with these materials than with the better known orientatable suspensions, such as myosin or vanadium pentoxide.

Bawden *et al.* (1936) observed that well oriented gels of plant virus could be prepared by cautiously drying a film of virus situated between a microscope slide and a cover-glass held abour 1/2 mm. from it by glass distance pieces. If the slide is held with the parallel distance pieces vertical, drying takes place almost exclusively along the bottom edge of the film. Under these conditions

a 1 per cent. solution of the SSS gives rise to a birefringent rod of the partially dried [PLAPS], which gives perfect extinctions under the polarizing microscope. This phenomenon is shown most strongly by the least modified preparations. Agents which either disaggregate the molecule without removing the loosely-bound phospholipid (PL1), for example, sodium dodecyl sulphate, or those which remove PL1 without causing complete disaggregation, for example, solvents, cause a diminution in the birefringence of the drying jelly. These agents cause an even more marked diminution in the anisotropy of flow of a solution of the modified [PLAPS]. Material that has lost PL1 and that has also been completely disaggregated shows no trace of birefringence under any of the conditions tried.

Solutions of [PLAPS] suffer no apparent change when stored at 0° C. for several weeks or even months. Bacteria grow in solutions kept without sterile precautions, but only to a very small extent. The solutions must, however, be protected from both drying and freezing, for, in either event, a considerable proportion of the SSS becomes insoluble and part dissociates from the PL1. If a 0·5 per cent. solution is either dried or frozen and is then dissolved or thawed and centrifuged, the insoluble material separates as an oily brown layer. This may be got into solution, *i. e.* into such a state that it cannot be sedimented by centrifuging for 1 hour at 3500 r.p.m., either by repeatedly suspending in water and centrifuging out what has not dissolved, or else by dissolving the oily material in 30–40 per cent. alcohol and then diluting. Solutions obtained in this way from insoluble material have appreciably different physical properties, and give serum end-points from 1 : 200,000 to 1 : 2,000,000. In spite of this variation, they all give rise to equally active preparations when disaggregated by acetic acid or by the action of sodium dodecyl sulphate. The variations are presumably due to differences in particle size or configuration, for the analytical figures on this dried material do not differ appreciably from those on [PLAPS]. The material that has been got into solution by the methods described can be sedimented to a large extent by 6 hours' centrifugation at 3500 r.p.m. ; at 12,000 r.p.m. sedimentation is complete in 10 minutes. The mechanism of this artificial condensation of [PLAPS] and the properties of the condensed material have not been investigated further, but enough has been done to show that it is unwise to attempt to prepare SSS in its least modified form from dried material.

After treatment with solvents, such as alcohol or alcohol and ether, the SSS can still be sedimented, but more slowly than before. It has been pointed out that freezing and drying give rise to products more easily sedimentable than [PLAPS] ; it is therefore essential to exercise caution in choosing a standard [PLAPS] for comparison of sedimentation rates, and the final choice is necessarily somewhat arbitrary, and depends on criteria such as the character of the precipitate with antisera and the homogeneity that is shown on the centrifuge. Ninety per cent. of a 5 mg. per c.c. solution of [PLAPS] can be sedimented in an Ecco Blitz angle centrifuge in half an hour at 14,000 r.p.m. The fluid column used in these experiments is 4 cm. deep, and the mid-point is 6·5 cm. from the axis of rotation. After removal of the PL1 90 per cent. sedimentation is only attained after 2–3 hours, centrifuging under these conditions.

Analyses on dried [PLAPS] need not be given in detail, for it differs analytically from AP only in the presence of the lipoid mixture that we call PL1 and in the presence of S. The nitrogen content of [PLAPS] is 4–5 per cent., whereas that of the dried bacteria is about 12 per cent. This fact, when taken in conjunction with the absence of microscopically recognizable bacteria or gross bacterial fragments in [PLAPS] solutions, rules out the possibility that [PLAPS] is the result of an indiscriminate break-up of the whole bacterial body. Total carbohydrate estimations cannot be carried out on [PLAPS] by the method described later, because the PL1 renders the solution too turbid to be matched in a colorimeter. There is, however, no reason to think that [PLAPS] contains any carbohydrate except that present in the component of it called AP. A little of the lipoid mixture, PL1, can be extracted from the dried material by solvents, such as chloroform or ether, but most of it cannot be extracted in this way. It may be removed completely by running a solution of the antigen into ten times its volume of a mixture of equal parts of alcohol and ether containing 0·5 per cent. of concentrated hydrochloric acid. The defatted [PLAPS] can be centrifuged off after half an hour, and if it is redissolved and reprecipitated in the same way, negligible amounts of material are found in the second extract. This method has been used for the routine analysis of all fractions of SSS; 0·5 c.c. of a 1 per cent. solution is added to 5 c.c. of the solvent mixture and, after centrifuging, the fluid is evaporated down and incinerated for phosphorus estimation by the method described later. This gives the PL1 phosphorus; phosphorus is bound in the antigen in two other ways besides PL1. These we call PL2 and labile phosphate, and they can be determined after hydrolysis of the material insoluble in the solvent mixture by methods to be described in a later paper. In a good sample of [PLAPS] there is 0·2–0·25 per cent. of phosphorus in the PL1 fraction, and about 20 per cent. of its dry weight is extractable by solvent in this way. Some properties of the lipoid fraction are summarized below.

DEGRADATION OF [PLAPS] TO AP.

It is obvious from the method of isolation that [PLAPS] can be centrifuged out or precipitated repeatedly without any change in its chemical or physical properties. It is also unaffected by boiling for short periods in neutral solution, by incubation with trypsin preparations or by exposure to dilute acids and alkalis. As might be expected, exposure to strong acids or alkalis or to hot dilute acids or alkalis causes hydrolysis, and the stages in this process will be described later. The changes that are brought about by organic solvents or by surface active substances, such as sodium dodecyl sulphate, are of greater interest and can be followed by serological, microscopical and physical or chemical methods.

Two chemical changes which do not lead to a loss of the ability to precipitate specific antisera have been recognized as taking place during the process of degradation: the loss of the phospholipid mixture PL1 and the loss of the substance S responsible for the Sakaguchi reaction that is given by [PLAPS]. The removal of PL1 by one precipitation with an acid alcohol-ether mixture has

already been described, but it is also removed by four or five precipitations with neutral alcohol in the presence of a trace of salt or by two or three precipitations with acid alcohol. The substance S was not removed by these treatments, but remained in the supernatant when four volumes of acetic acid were added to an aqueous solution of [PLAPS] and, after standing for half an hour, the SSS was precipitated by the addition to this fluid of 1·3 times its volume of alcohol. After two or three precipitations in this way AP generally gives no detectable Sakaguchi reaction when tested under conditions where 0·003 mg. of arginine show up clearly in the presence of 4 mg. of antigen. It has occasionally proved impossible to remove the last traces of Sakaguchi reaction from batches recovered from residues, but this difficulty is not experienced in the course of a normal preparation.

There is at present no evidence as to the connection between the liberation of the substance S and the process of degradation, but it can be shown that degradation to this extent is not a readily reversible process. A solution of [PLAPS] in 80 per cent. acetic acid was distilled *in vacuo* at low temperature, with the addition of water from time to time to keep it from going dry, until most of the acetic acid had been removed. The resulting solution was nearly clear, *i. e.* like AP, although it contained both PL1 and S. From this solution material sedimented as slowly as from a solution of AP, whereas a solution of [PLAPS], to which as much acetic acid had been added as remains in the AP solution after distillation, sedimented normally.

From the observations made on the centrifugation of [PLAPS] it is not possible to calculate a sedimentation constant because of the angle of the tubes, and the turbulence necessarily established in them under the conditions of centrifuging. Furthermore, if a sedimentation constant were calculated, it would not be interpretable, since the particles of [PLAPS] are certainly not spherical. It is, however, clear that these particles are of the same order of size as the more thoroughly studied plant virus preparations. AP sediments slowly at 14,000 r.p.m., and measurements have been made on it in the ultra-centrifuge by Mr. J. StL. Philpot. He finds that the preparations are poly-disperse, and that the chief component has $S_w\ 20 = 50 \times 10^{-13}$. This sedimentation constant, if we make the usual assumptions, corresponds to a molecular weight of about one million.

THE PROPERTIES OF AP.

Degraded SSS, free from PL1, that has been purified and dried while frozen, is a soft white coherent mass with the consistency of cotton-wool. When dried over phosphorus pentoxide *in vacuo* it is, like many large molecules, intensely hygroscopic, and absorbs 5–10 per cent. of its weight of water; after absorbing this much water it is stable in moist air and does not become sticky. Part of any dried purified preparation is always easily soluble in water but part is insoluble, and the relative proportions of these two parts depends on the previous treatment and, presumably, on the condition of aggregation that has been reached during the drying. About 90 per cent. is generally soluble, and the insoluble part can be got into solution by adding alkalis, or by moistening the powder with a little alcohol or glacial acetic acid before adding water. No

chemical or serological differences have been noticed between material that goes into solution immediately and material that needs to be treated in these ways.

Solutions of AP are clear enough for polarimetric readings to be made on neutral 0·5 per cent. solutions in a 2 dm. tube. The specific rotation is $\left[+43°\right]^{D}_{16°}$, whether the material is in water or in $N/20$ hydrochloric acid, and it is unaffected by heating with acid for periods insufficient to cause the fluids to become opalescent through separation of PL2.

Dried material has the following analytical composition: C., 48–50 per cent.; H., 7·5 per cent.; N., 5·4 per cent.; S., 0·1–0·2 per cent.; P., 0·5–0·6 per cent.; volatile acid estimated as acetyl, 14 per cent.; carbohydrate estimated as glucose, 14 per cent.; ash, 2–4 per cent. Phosphorus estimations were carried out by a method based on that of Kuttner and Lichtenstein (1932), acetyl estimations by the method of Elek and Harte (1936), and carbohydrate by a modification (Pirie, 1936) of the Tollens orcin method. The other estimations are by the usual Pregl methods. The metal present in the ash has not been studied, but most of the phosphorus of AP is found in the ash after incineration.

From the method of preparation it is clear that AP is soluble in water over the whole pH range, although it is readily absorbed by any other material which is being precipitated by acid; it is soluble in acetic acid if the concentration does not exceed 90 per cent. It is easily precipitated by alcohol (methyl or ethyl) and by acetone in the presence of an acid or other electrolyte, and it is precipitated by ammonium sulphate at room temperature when the concentration is raised to 36–37 per cent. of saturation. At 0° C. it is necessary to raise the concentration to 45 per cent. of saturation before precipitation takes place. AP is also precipitated by magnesium sulphate, but this process is of little value as a means of isolation, for the precipitate has so nearly the same specific gravity as the suspending fluid that the mixture does not separate on centrifugation and filtration is very slow. There are various precipitants, such as neutral or alkaline mercury salts, colloidal iron, tannic acid, basic lead acetate, copper or zinc salts in neutral or slightly alkaline solution and phosphotungstic acid in the presence of acids, which precipitate the AP quantitatively. They have not, however, been found useful in purification, either because they precipitate the contaminants under the same conditions, or else because of the difficulty experienced in recovering the AP from the precipitate. This objection also applies to the use of Kieselguhr and alumina, which absorb AP readily. Uranyl nitrate, neutral lead acetate, tungstic acid, ferric chloride, safranine and neutral silver or lanthanum salts do not give precipitates with pure preparations, but they can frequently be used to separate some impurities from crude preparations.

Little effect seems to be produced on AP by boiling in neutral solution, by drying, or by incubation with the commercially available enzyme preparations or with lysozyme. The material is substantially protein-free, *i. e.* it fails to give a biuret reaction or colour reactions for tyrosine or tryptophane under conditions where a clear reaction is given by a preparation mixed with 3 per cent. of caseinogen.

All cultures have been grown on media prepared with liver extracts; it is therefore impossible to exclude the possibility that the preparations are contaminated with materials derived from the liver, although we see no reason to suspect such contamination. The possibility that any of the properties described should be due to contamination with agar or with material derived from the trypsin preparations used can, however, be excluded, for preparations made without the use of trypsin or from cultures not grown on agar have had the same properties.

THE FRACTION S.

All samples of [PLAPS] prepared by sedimentation on the high-speed centrifuge give equally intense Sakaguchi reactions. The test was carried out under conditions based on those of Weber (1930): to 2 c.c. of the neutral fluid to be tested 0·4 c.c. of 10 per cent. sodium hydroxide and 0·4 c.c. of 0·02 per cent. alcoholic *a*-naphthol were added and the mixture cooled in ice. Three drops of a solution of sodium hypobromite made by adding 0·2 c.c. of bromine to 30 c.c. of 5 per cent. sodium hydroxide are added and, after 15 seconds, 0·4 c.c. of 40 per cent. urea. The fluid can be compared with a standard in a colorimeter, but it is more convenient to measure the colour on a tintometer in a cell 1 cm. deep, for the colour fades in a few minutes. Under these conditions 0·02 mg. of arginine correspond to 4 red units and 1 mg. of casein to 5–6 R.U.; 2·5 mg. of antigen give 1 R.U. and a little yellow. Care is needed in using the test, for it is inhibited by many substances, including an excess of protein; a test is taken as negative when 0·003 mg. of arginine hydrochloride give a detectable colour in the presence of the amount of the material that is being tested that is recorded as giving no colour.

The removal of this substance from the antigen has already been described as a change which takes place during the disintegration of the molecule with acetic acid. A similar disintegration with removal of S takes place when a solution of the antigen is shaken with chloroform mechanically for some hours. The process is slow, and if the aqueous solution is centrifuged at high speed before the disintegration is complete, it is found that the sedimentable material still gives a Sakaguchi reaction of nearly the usual intensity, *i. e.* 1 R.U. with 3·5 mg., whereas 5 mg. of the material that is not sedimented will give no colour.

A more detailed description of S will be published later, but a summary may be given here. Part at least of the S fraction can be isolated by distilling the alcohol and acetic acid mother liquors from the degradation of antigen to dryness *in vacuo* and redissolving in a little acetic acid. From the solution a substance can be precipitated by the addition of ether, which is free from lipoid and is soluble in dilute alkalis. This still contains some AP; it can be purified further by dissolving in $N/10$ HCl and adding 3 volumes of alcohol. The AP but relatively little of the S precipitates, and the S can be recovered from the supernatant by the addition of more alcohol. When prepared in this way it contains 9 per cent. of nitrogen and gives a biuret, Millon and xanthoproteic reaction, besides the strong Sakaguchi reaction (3–5 R.U. per mg.). The substance S is, therefore, apparently either a protein or a peptide; this idea is supported by the character of the absorption spectrum, for S absorbs

U.V. light at 270 mμ. as strongly as most protein solutions and much more strongly than AP. The yield of this material is as yet only 5 per cent. of the weight of the antigen used, and thus accounts for rather less than half the Sakaguchi reaction given by the antigen. It would be reasonable to assume that this material is simply bacterial protein carried through mechanically, were it not for the fact that more than fifty preparations of native antigen and of lipoid-free antigen made by careful centrifuging and centrifugal refractionation have contained the same amount of the S substance, and that this amount is unaffected by any treatments, such as boiling or incubation with proteolytic enzyme preparations, which do not affect the sedimentability of the antigen. For these reasons it seems necessary to assume that this substance also is an integral part of the native bacterial antigen.

THE LIPOID MIXTURE PL I.

Chemical study of the lipoid fraction, PL 1, is complicated by the fact that the bacteria contain large amounts of lipoid that is not apparently associated with the antigen. If the bacterial mass is extracted by methods similar to those used by Anderson (1927) a mixture of both types of lipoid results, and no information is gained about PL 1. The lipoid fraction that is isolated by treating purified [PLAPS] solutions with alcohol-ether mixtures containing hydrochloric acid consists of a phosphorus-free, acetone-soluble fat, and at least two phosphorus and nitrogen-containing lipoids, which are insoluble in acetone but soluble in chloroform, benzene and in alcohol-ether mixtures. This phospho-lipoid fraction can be separated into a number of components by crystallization from alcohol, and about half of the total phosphorus of PL 1 can be separated as a substance insoluble in hot alcohol and containing about 2 per cent. of phosphorus. All these materials can be prepared in the form of soft masses which appear to be crystalline under the polarizing microscope, but there is as yet no reason to look on any fraction as a pure substance.

In succeeding papers of this series the serological behaviour of the antigen and its behaviour in the living animal will be described, and evidence will be presented bearing on its chemical constitution. The general summary and all discussion of the results will be postponed until these other parts have been published.

SUMMARY.

Methods are described for the isolation of the antigen of *Br. melitensis* in two clearly distinguishable states of aggregation. When in the state in which it has the greater particle size the antigen is associated with a mixture of lipoids and phospholipoids and with a protein-like material. The physical properties of all these substances are described, and the chemical properties of the lipoids and of the protein-like material are summarized.

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A CHEMICAL CHANGE OCCURRING DURING THE LYSIS OF *BACT. COLI* BY BACTERIOPHAGE.

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VERY little is known of the chemical changes that take place when bacteria are lysed by phage. Hetler and Bronfenbrenner (1928) reported that lysed cultures of various bacteria contained more NH_2 nitrogen than control cultures, and concluded that the increase came from hydrolysed bacterial protein. Northrop (1938), using a turbidity method to estimate total and water-insoluble protein, considered that during the lysis of *Bact. coli* unhydrolysed protein was liberated, but that during the lysis of some other bacteria hydrolysis of protein and lysis were simultaneous. These two reports are the only ones giving positive results of chemical changes during lysis of bacteria by any phage.

Meyer *et al.* (1936) found that purified lysozyme hydrolysed certain mucoids from egg-white or from sarcinæ susceptible to lysozyme. The hydrolysis could be followed by measuring the production of reducing sugars, and Meyer considered that this hydrolysis of carbohydrate by lysozyme caused disintegration of the bacterial cell. Non-protein nitrogen and inorganic phosphorus were also formed during the action of lysozyme on heat-killed sarcinæ.

Dubos (1937*a*) has recently separated a similar enzyme from pneumococci. In high concentration it lyses heat-killed cells. In lower concentrations, or after partial purification, it changes such cells from the Gram-positive to the Gram-negative state. This change in staining powers was prevented by a previous incubation of the enzyme with a polysaccharide acid from vitreous humour or the umbilical cord. Meyer, Dubos and Smyth (1937) found that the enzyme from pneumococcus would hydrolyse such polysaccharide acids under the conditions in which it brought about the staining change in pneumococci or the lysis of the cells, and they conclude that the enzyme is responsible for all these changes. The enzyme removes from the bacteria the property of stimulating the formation of precipitating antibodies from the type-specific polysaccharide (Dubos, 1937*b*).

A common factor, therefore, in the lysis of two different bacteria is thought to be the hydrolysis of a polysaccharide acid, and the question naturally arises whether lysis of bacteria by phage is brought about by an enzyme of this type.

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EXPERIMENTAL METHODS.

In all the experiments to be described phage WLL and *Bact. coli* 88 and 14 have been used. They were obtained from the late Dr. Schlesinger and were the organisms used by him in his work on the purification of phage (Schlesinger, 1933, 1934). Phage WLL has been grown on *Bact. coli* 88 when small quantities were needed, but for the purposes of concentration it has been grown on *Bact. coli* 14 in a medium described by Schlesinger in which lactate is the sole source of carbon. The method of concentration of phage by precipitation with $(NH_4)_2SO_4$ will be described elsewhere.

Phage titre was estimated by the plaque method. The counting method was a modification of the surface viable count of Miles and Misra (1938), in which the drop areas are of phage rather than of bacteria. The results recorded are the means of plaque counts in 6–12-drop areas, reduced to the nearest integer. Calculations of the statistical significance of differences between counts are unnecessary, since the relevant counts differ grossly by 10–1000-fold.

Growth and lysis of bacteria were estimated by a turbidity method. Turbidity was estimated photo-electrically (Clifton, Mueller and Rogers, 1935). Within the limits of concentration used the log. per cent. light transmitted is directly proportional to the concentration of organism. A reference graph was constructed using washed suspensions of *Bact. coli* 88.

Reducing sugars were estimated by Giragossintz, Davidson and Kirk's (1936) method. The ferrocyanide formed was titrated with cerium sulphate in acid solution ; 0·2 per cent. xylene cyanole FF was used as an indicator. The cultures were deproteinized with cadmium (Fujita and Iwatake, 1931) ; 3·5 ml. $CdSO_4$ (13 g. per litre of 0·06 *N* H_2SO_4) *plus* 0·75 ml. of 0·62 *N* NaOH were added to 2·0 ml. of the culture. The precipitate was centrifuged off and 2·0 ml. of the clear centrifugate was used for each estimation. Duplicates of the samples and controls of the reagents were always done. One ml. of the cerium sulphate used was equivalent to 0·1 mg. of glucose.

Polysaccharide was estimated colorimetrically with orcin (Pirie, 1936). The total carbohydrate of the precipitate obtained at pH 4·5 with 80 per cent. C_2H_5OH or with 50 per cent. saturation with $(NH_4)_2SO_4$ was used as a measure of the polysaccharides of the cell. Alcohol could not be used as precipitant with broth cultures of *Bact. coli* 88 as it precipitated carbohydrate from the medium alone, and it was with these cultures that $(NH_4)_2SO_4$ was used.

Effect of Phage on Non-viable Bacteria.

Many experiments were made to see if concentrated phage preparations having titres of between 10^{11} and 10^{12} particles per ml. contained an enzyme like lysozyme. But heat-killed, chloroform-killed and acetone-dried bacteria were all unaffected when incubated with concentrated phage suspensions. No lysis, decrease in carbohydrate or production of reducing sugars from any bacterial substrate could be demonstrated.

Since no success was obtained using unnatural substrates for phage, recourse was made to viable *coli*. It is difficult, when using this as substrate, to determine whether any change produced is due to bacterial or to phage enzyme.

Furthermore, a chemical change started by phage might be carried further by bacterial enzymes and therefore recognizable end-products might be lacking. These facts were borne in mind when interpreting results.

Changes in Carbohydrate During Lysis.

Experiment 1.

Bact. coli 14 was grown overnight in Schlesinger's lactate medium, 100 ml. per Roux bottle. Ten ml. phage of titre 2×10^{11} was then added to one culture and 10 ml. boiled phage to the control. Five ml. samples were pipetted out immediately and at later intervals and the turbidity and reducing sugar content of the two cultures were estimated (Fig. 1).

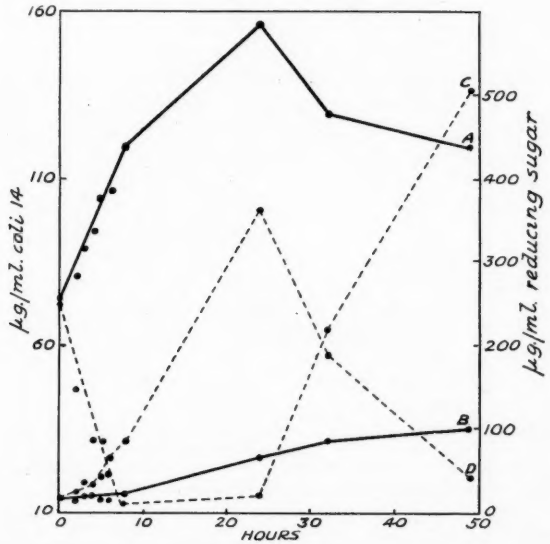

FIG. 1.—Production of reducing sugars during lysis of *Bact. coli* 14. Curve A, control : µg. *Bact. coli* 14 : ml. Curve B, control ; µg. reducing sugars : ml. Curve C, phage ; µg. *Bact. coli* 14 : ml. Curve D, phage ; µg. reducing sugars : ml.

During lysis of *Bact. coli* 14 there is a large increase of reducing sugars in the medium. In normal cultures of this strain growing on this lactate medium, the reducing sugar content remains roughly constant during the most active growth period. In cultures where phage is present reducing sugars continued to increase after lysis was over, judged turbidimetrically, but when overgrowth of the bacterium took place the concentration fell again.

Changes in polysaccharide were then estimated to see whether these ran parallel to the course of lysis. For if the disintegration of the bacterial cell

is caused by hydrolysis of polysaccharide, the primary cleavage may be more important than the final production of reducing sugars.

Experiment 2.

Bact. coli 88 was grown in 30 ml. of meat broth in boiling tubes until the medium was definitely turbid. Four ml. of a concentrated suspension of phage of titre 1×10^{11} was added to one tube. Samples were taken at once from this tube and the control, and at various intervals during lysis. One ml. of each sample was precipitated with 1·0 ml. of saturated $(NH_4)_2SO_4$ at pH 4·5 in conical graduated centrifuge tubes. The precipitate was centrifuged off and drained ;

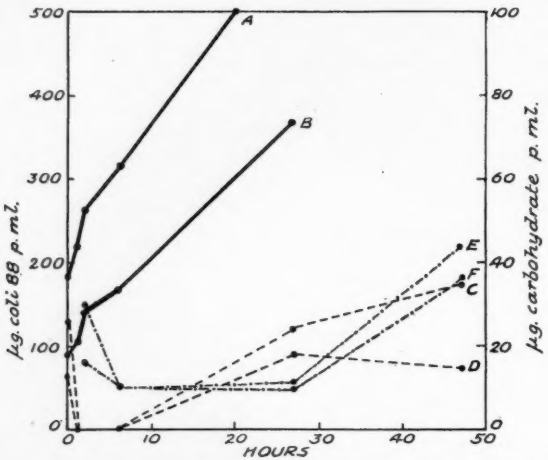

FIG. 2.—Decrease of precipitable carbohydrate during lysis of *Bact. coli* 88. Curve A, control ; μg. *Bact. coli* 88/ml. Curve B, control ; μg. carbohydrate/ml. Curve C, phage ; μg. *Bact. coli* 88/ml. Curve D, phage ; μg. carbohydrate/ml. Curve E, 2-hour mixture of control + phage ; μg. *Bact. coli* 88/ml. Curve E, same ; μg. carbohydrate/ml.

0·2 ml. H_2O + 1·8 ml. of 0·2 per cent. orcin in 66 per cent. H_2SO_4 was added. The tubes were heated, together with a standard tube containing 0·2 ml. of a solution of glucose 250 μg./ml. + 1·8 ml. orcin reagent, in a water-bath for 10–15 min. They were then cooled, diluted to 10 ml. and the amount of carbohydrate estimated colorimetrically (Pirie, 1936). The turbidity of 1/4 dilutions of the cultures was estimated photo-electrically. After 2 hours 10 ml. of the control and 10 ml. of the lysed culture were mixed and the turbidity and carbohydrate content of this third tube was estimated at intervals (Fig. 2).

During the lysis of either *Bact. coli* 88 or 14 the polysaccharide content of the culture decreased, but it did not continue to do so after lysis was over. Overgrowth of the bacterium caused an increase again, showing that the carbohydrate estimated was part of the bacterial cell. During growth of the control cultures there was a steady rise in polysaccharide for the first 24 hours or more.

The decreases in polysaccharide during lysis are small, but duplicate estimations showed that they were outside the experimental error. There was no relation between the amount of reducing sugars produced during lysis of *Bact. coli* 14 and the amount of polysaccharide removed. During lysis many enzymes must be active, and the complexity of the process affords a possible explanation of this lack of balance. In a lysing culture both growth and lysis are proceeding and, if growth involves production of carbohydrate mainly from the lactate of the medium rather than from any reducing sugars present, there will be a gradual accumulation of these in the medium unless they are rapidly oxidized. Such an accumulation does in fact take place as shown in Fig. 1.

The change in reducing sugars during lysis of *Bact. coli* 88 could not be estimated, as the reducing sugar content of the broth on which this strain was grown was initially too high for small differences to be demonstrated. Table I gives a summary of the results with both strains.

TABLE I.—*Production of Reducing Sugars and Decrease of Total Precipitable Carbohydrate during Lysis. Summary of Results. The Conditions of Growth the same as in Experiments* 1 *and* 2.

	Organism.	Time (hrs.).	Decrease in carbohydrate (μg./ml.).	Increase in reducing sugars (μg./ml.).
1	14	8	− 7	+ 17
		11	− 9	+ 47
2	14	9	− 6	+ 23
3	14	8	− 27	..
4	14	8	..	+ 95
5	14	19	..	+ 94
6	88	9	− 27	..
7	88	5½	− 9	..
8	88	3	− 6	..

Chemical Changes During Autolysis.

Experiments were made to see whether these changes in carbohydrate occurred only during lysis, in which case they might possibly be considered to be due to the action of enzymes contained in phage, or whether they occurred also during autolysis of the bacterial cell. Figs. 1 and 2 show that they do not occur during normal bacterial growth. I found that during autolysis of both these strains of *Bact. coli* the same chemical changes took place. Neither strain autolyses rapidly or completely, the most obvious change being one in staining power. Thionin, which stains the normal bacteria purple, does not stain those which have been allowed to autolyse for 12 hours, although the cells are still intact and can be stained by Mann's methyl-blue-eosin stain. Thionin stains mucin purple (Kelly and Miller, 1935), and it seems possible that this change in staining power is related to the hydrolysis of the polysaccharide. In order to follow the change in reducing sugar during autolysis of *Bact. coli* 88, *M*/500 sodium iodoacetate was added. This prevents the further breakdown

of reducing sugars without inhibiting their production, or the staining change in the bacteria. Much more concentrated suspensions of bacteria could be used in these experiments than when investigating lysis by phage and therefore the chemical changes are correspondingly larger (Table II).

TABLE II.—*Production of Reducing Sugars and Decrease in Total Precipitable Carbohydrate During Autolysis.*

	Organism.	Time. (hrs.).	Decrease in carbohydrate (μg./ml.).	Increase in reducing sugars (μg./ml.).	Iodoacetate.
1	88	20	— 328	+ 263	*M*/500
2	88	36	..	+ 275	*M*/500
		36	..	+ 31	..
3	14	22	..	+ 183	*M*/500
		22	..	+ 130	..
4	14	20	— 106	+ 48	..

Bact. coli 14 and 88 grown overnight in appropriate media, centrifuged and the sedimented bacteria made up in a small volume of water. This concentrated suspension was incubated at 37° C. in the presence of toluene or of *M*/500 iodoacetate; 0·5 ml. samples were used for estimation of reducing sugars and 0·2 ml. for precipitable carbohydrate.

In lysis and autolysis of these two strains of *Bact. coli*, therefore, the same chemical changes occur in the carbohydrate fraction of the bacterial cell, and the question arises whether these are in any way responsible for its disintegration and whether lysis is brought about by bacterial enzymes. If lysis is caused by bacterial enzymes there seems no reason why growth of the bacterium is necessary for it, nor, except from quantitative considerations, is there any reason why growth of phage should be directly connected with lysis. The relation of growth of bacterium, and of phage, to lysis has never been clearly defined. In some cases lysis with no growth of phage has been reported, and in some cases growth of phage with no lysis (Doerr and Berger, 1923; Kreuger, 1936). It may be that phage inadvertently causes the destruction of its host, possibly through the action of that host's own enzymes.

Lysis in the Presence of Growth Inhibitor.

Lysis can be separated both from growth of bacteria and from growth of phage by allowing it to proceed in the presence of iodoacetate. This substance inhibits bacterial growth without making the cells non-viable when transferred to fresh medium. Iodoacetate does not inhibit hydrolysis of polysaccharide during autolysis as we have seen. Lysis is also not retarded—in fact it is sometimes accelerated, as no bacterial growth is taking place. During lysis the titre of phage falls until by the time lysis is complete it may be 1/20th or less of the original titre of the culture. This fall in titre may be due to the reaction of phage with " phage inhibitor " released from the lysed cells. It is not due to any effect of iodoacetate on phage itself, as control mixtures of phage and iodoacetate show no estimatable fall in titre during the same period of

incubation. Unless one considers that a much greater amount of " phage inhibitor " is released from the cells during lysis in the presence of iodoacetate than under normal conditions, one must consider that lysis takes place here with no phage growth (Table III).

TABLE III.—*The Effect of Iodoacetate on Lysis and on Titre of Phage.*

Mixture.*	*Bact. coli* 88. μg/ml. after—			Phage titre after:		
	0 hr.	1 hr.	4½ hr.	0 hr.	1 hr.	4½ hr.
"88" + P	88	..	20	..	..	..
"88" + P + IA	44	..	22	5×10^6	..	$<6 \times 10^2$
IA + P	..	..	..	2×10^7	..	2×10^7
"88" + P	57	..	15	..	..	..
"88" + P + IA	69	..	29	$>1 \times 10^8$	..	2×10^6
"88" + IA	65	..	57	..	..	..
"88" + P	98	..	19	1×10^7	..	1×10^{10}
"88" + P + IA	103	..	69	7×10^6	..	$<4 \times 10^5$
IA + P	..	..	..	1×10^7	..	1×10^7
"88" + P + IA	65	31	12	$>1 \times 10^8$	3×10^7	2×10^6

Bact. coli 88 was grown in tubes of 30 ml. of meat broth until a definite turbidity was visible. A final concentration of *M*/1000 sodium iodoacetate and 3·0 ml. of stock phage was added to one tube. Iodoacetate and phage controls were also set up. Samples were taken immediately and at intervals during lysis for estimations of turbidity and titre. The titre is expressed as particles per ml.

* "88" denotes *Bact. coli* 88; P, phage and IA, iodoacetate.

Lytic Enzymes.

D'Herelle (1926) first suggested that a lytic enzyme was secreted by phage and more recently Sertic (1929) and Schuurman (1936) have brought forward evidence in favour of this view. They consider that the enzyme diffuses out of the plaque into the surrounding agar. Phages differ greatly in their power to produce this enzyme. It will lyse heat-killed bacteria and causes a surface change in living bacteria, which renders them non-agglutinable by trypaflavin (Sertic, 1936). The evidence is clear that an enzyme with these properties is produced, but it is not certain that it is an enzyme produced by phage rather than by the bacterium. Sertic (1929) states that water or glycerin extracts of bacteria fail to give a lysin, but it is possible that other methods of extraction might have been successful. The evidence in favour of the view that the lysin is of bacterial origin is that lysis of bacteria resistant to phage can be brought about in lysing cultures of phage-susceptible organisms. Wollman (Wollman and Wollman, 1933, 1936; Wollman, 1934) showed that heated bacteria related to the susceptible strain were lysed if they were added to a

culture of this strain *plus* phage. He also showed that a fresh filtrate of a lysed culture of staphylococci would lyse staphylococci which were resistant to the phage used to obtain the original lysis. Wollman failed to obtain lysins or secondary lysis of dead bacteria with *Bact. coli*. He concludes that phage stimulates autolysis. Bronfenbrenner and Muckenfuss (1927) obtained an enzyme from both normal and lysed cultures of staphylococci which would lyse dead bacteria. The balance of evidence seems in favour of the view that the lysins so far described are bacterial enzymes.

Enzyme from Bact. coli.

An enzyme preparation, from *Bact. coli* 88, capable of removing carbohydrate from heat-killed cells could be obtained easily. The supernatant fluid from a 24–48-hour culture was precipitated by 40 per cent. saturation with $(NH_4)_2SO_4$ at pH 5·0. The precipitate was centrifuged off, suspended in water, neutralized and dialysed. The precipitate which came out during dialysis was discarded and the active enzyme found in the supernatant. When heat-killed *Bact. coli* was incubated with this enzyme there was a loss of precipitable carbohydrate, up to 1/3 of the total being removed, but there was no estimatable change in reducing sugars, showing that hydrolysis of the carbohydrate was far from complete. At the same time the cells lost the power of being stained with thionin. It would be interesting to know whether preparations of lysins, which cause a surface change in bacteria, contain enzymes of this type. The one extracted from *Bact. coli* 88 is unstable, and sometimes became inactive after 24 hours in the ice-chest (Table IV).

TABLE IV.—*Effect of Bacterial Enzyme on Carbohydrate of Heated* Bact. coli.

	Time (hrs.).	Decrease in carbohydrates (μg./ml.).	Total precipitable carbohydrate (per cent.).
1	1½	— 65	12
	2½	— 39	25
2	3	— 42	14
3	3	— 38	30
4	18	— 85	26
5	20	— 159	29

Bact. coli 88 was grown overnight, centrifuged down and the sediment made up in a small volume of water and heated rapidly in a water-bath to 90°–95° C. Thick suspensions were used. Equal volumes of bacterial suspension and enzyme were mixed, and phosphate buffer pH 7·4 to a final concentration of *M*/50 was added. If the experiment was to last more than three hours a drop of toluene was added; 0·1–0·5 ml. samples were taken for estimation of precipitable carbohydrate by the usual method. There was no detectable increase in reducing sugars in 0·5 ml. samples whether iodoacetate was present or not. Controls of boiled enzyme *plus coli* and of the enzyme *plus* water were carried out. The decreases in carbohydrate shown are the total changes *minus* any changes occurring during incubation of such controls.

Effect of Iodine.

Both lysozyme and the autolytic enzymes of pneumococcus are inactivated by iodine (Meyer *et al.*, 1936 ; Dubos, 1937*a*) and partly re-activated by reduction with sulphite. A study was made of the effect of iodine on lysis and autolysis of *Bact. coli* 88. It is easily made non-viable by iodine, the concentration required depending on the amount of bacteria present. Such non-viable *Bact. coli* still autolyses gradually even when killed with $N/100$ I_2. Treatment with sulphite makes little difference to the rate of autolysis, though, after treatment with the higher iodine concentrations, there was a tendency for the re-reduced *coli* to autolyse more rapidly. During autolysis there was a decrease in precipitable carbohydrate and a smaller increase in reducing sugars in the presence of iodoacetate. Such non-viable *coli* are more readily disintegrated in the presence of phage, whatever concentration of iodine had been used to render them non-viable. Since the enzyme which hydrolyses carbohydrate is not completely inactivated by any of the concentrations of iodine that have been used, it is impossible to decide by this method whether its activity is necessary for lysis or not.

DISCUSSION.

So little is known of the chemical changes occurring during lysis that it seemed worth while to present these results, although there is no evidence that the chemical change described is necessary for the disintegration of the bacterial cell. The specificity of a phage is determined by the antigenic structure of the bacterium (Burnet, 1934; Kreuger, 1936), and one point of attachment of a phage to the cell seems in many cases to be a serologically active polysaccharide (Bruce White, 1936 ; Burnet and Freeman, 1937 ; Craigie and Brandon, 1936). In this connection the results obtained by Bruce White (1937) are especially interesting. He found that a weak cholera phage could be made much more active if 1/25 egg-white was added to the medium, and he concluded that this activation was brought about by lysozyme. By culturing different strains of cholera in a medium containing egg-white many were shown to be lysogenic. This phage is known to be inactivated by the polysaccharide of S, R and ρV cholera strains. In this case therefore there is a direct connection between the activity of the phage and the cell polysaccharide. Since lysozyme hydrolyses certain polysaccharides, such as those from the vitreous humor and Group A streptococci, it is possible that the increase in activity of the phage is due to the hydrolysis by egg-white lysozyme of the polysaccharides to which the phage is bound on the cell surface.

SUMMARY.

A decrease of precipitable carbohydrate occurs concurrently with the lysis of two strains of *coli* by phage WLL. An increase of reducing sugars in the medium could be detected during the lysis of *Bact. coli* 14. This increase continues after lysis is over. Similar changes occur in autolysis of these two

strains. An enzyme could be separated from normal *coli* which removed carbohydrate from heat-killed bacteria. No such enzyme was present in concentrated phage preparations. Lysis can take place in the presence of iodoacetate which does not inhibit these enzymes, although it stops bacterial growth. During this lysis the titre of the phage falls continuously.

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SURVEY OF PAPERS.

HENDERSON has studied the immunological properties of antigens extracted from typhoid bacilli by diethylene-glycol. It would appear that structural changes in the antigens occur during the process, which leads to a loss of function (p. 1).

HENDERSON finds that antiserum protects mice against typhoid infection more efficiently when the infecting dose is a small number of highly virulent organisms than when a large number of less virulent are injected. The efficiency of the protection is thus determined by the mass of bacterial antigen in the tissues rather than by the virulence of the strain used (p. 11).

ANDERSON AND OAG throw light on the mechanism by which mucin increases the virulence of certain bacteria. The subject of virulence is still very obscure, involving an interplay of invasive and toxic properties on the part of the micro-organism, and of the various modes of resistance by the host. The activity of mucin is shown to be due to the protein moiety, the carbohydrate fraction being inactive, although other carbohydrates such as agar and polysaccharides derived from the diphtheria bacillus have the same effect as mucin (p. 25).

KAPP gives spectrographic evidence in support of the view that a combined form of coproporphyrin which occurs in human urine is a zinc compound. She also records and discusses the ultra-violet absorption spectra of free coproporphyrin and of zinc coproporphyrin (p. 33).

EVANS, HAPPOLD AND HANDLEY have extended nutritional studies on *C. diphtheriæ* to cover the varieties known as "gravis", "mitis" and "intermediate". The nutrients found by others to be adequate for the strains tested are not adequate for the "intermediates". This variety requires, in addition, one or more of a number of inorganic salts (p. 41).

Previously McIntosh had shown that cell-free filtrates from Rous tumours, induced in fowls by tar-painting, were capable of inducing tumours in fowls in series. This result suggested the possibility that a virus was the active agent in the production of these tumours. Subsequently other workers have endeavoured unsuccessfully to pass chemically-induced tumours with cell-free filtrates. MCINTOSH AND SELBIE have now repeated and extended the work with a result similar to that of McIntosh, and give evidence in support of their view that a virus has a causal connection with these tumours (p. 49).

MILES has confirmed the hypothesis that the antigenic surface of smooth *Brucella abortus* and *melitensis* contains both A and M antigens, A being the major antigen in *abortus*, M that in *melitensis*. The ratio of major to minor antigen on the surface of the two species varies between 50 : 1 and 10 : 1. The rabbit responds to this mixture of antigens by the formation of antibodies, anti-A, anti-M, and apparently an anti-AM, bearing on one particle receptors for both antigens (p. 63).

MILES AND PIRIE publish part of an attempt to isolate from bacteria of the *Brucella* group the specific soluble substances responsible for the differences in the serological behaviour of the three species, *abortus*, *suis* and *melitensis*; it deals mainly with *Br. melitensis*. In the first part the preparation and physical properties of the antigen from smooth *Br. melitensis* are described. The antigen in its native state is serologically and chemically complex; it consists of a nitrogenous substance, analogous to the specific soluble substances of many bacteria, combined with a protein-like material and a mixture of lipoids (p. 83).

Lysozyme is a principle contained in various animal fluids which is capable of dissolving certain bacteria. It is now recognized that lysozyme preparations contain a carbohydrate-splitting ferment. A. PIRIE finds that during lysis of *Bact. coli* by bacteriophage a similar carbohydrate-splitting enzyme is active, but that the enzyme is not present in concentrated bacteriophage preparations. She concludes that growth, either of phage or of bacteria, is unnecessary for lysis to occur, but that the disintegration may be brought about by the bacterial enzymes (p. 99).

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